

Annual Progress Report

ONR Grant No. N00014-95-1-0463

MICROBIAL DIVERSITY  
A Summer Course at MBL

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1945-1946

## STAFF

### Co-directors:

Edward R. Leadbetter  
Abigail A. Salyers

### Course co-ordinator, assistant:

Angelica P. Seitz

### Post-doctoral teaching fellows:

Joel Dore  
Jorg Overmann  
Thomas Pitta

### Laboratory teaching assistants:

Michael Cerio  
John D'Elia  
Marion Leclerc

### Laboratory assistant:

Judith Whittier

### Guest lecturers; Directors, special laboratory techniques

Carl Bauer  
Robert Bullis  
Wm. Chesbro  
Paul Dunlap  
Robert Haselkorn  
Brian Howes  
Holger Jannasch  
Saundra Nierzwicki-Bauer  
Kenneth Noll  
Nadja Shoemaker  
Deborah Siegle  
Mitchell Sogin  
John Waterbury

Institutional affiliations, etc., of faculty and visiting speakers are found on the following six pages (Student/staff/faculty list).

## STUDENT LIST

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Crosby, Laurel  
Damgaard, Lars  
George, Alison  
He, Jiancai  
Hilario-Andrade, Elena  
Holliger, Christoff  
Hoskins, Dionne  
Lazar, Sara  
Miller, Eric  
Newman, Dianne  
Plugge, Caroline  
Sabl, Joy  
Schlekat, Christian  
Sroga, Grazyna  
Suarez-Sanchez, Paula  
Vettriani, Constantino  
Zannoni, Davide

Motylewski, Kim (a fellow in the Science Writers Program at MBL who participated in the course for the first three weeks during tenure of her stay at MBL).



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Affiliations, addresses of students will be found on the student/staff/faculty list.

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# MICROBIAL DIVERSITY GUEST LECTURERS

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# MICROBIAL DIVERSITY -- SUMMER 1995

## ACTIVITIES SCHEDULE

Lectures and laboratory in Loeb 208 (except those indicated [\*\*] which are in Candlehouse 105)

DATE / TIME	TOPIC	LECTURER
JUNE:		
S 11 20:00	GET-ACQUAINTED SESSION	
M 12 08:30	The course and its aims	E. Leadbetter &
10:00	Microbial diversity, an overview	A. Salyers
13:00	Microscopy I	T. Pitta
14:30	Microscopy II	"
16:00	Microscopy demonstrations	"
19:30	The "Volta" flame	In the field
T 13 08:30 **	Introduction to Woods Hole	H. Jannasch (WHOI)
10:00	Enrichment culture: theory & practice	E. Leadbetter
13:00	The Sippewissett Salt Marsh:	B. Howes &
	Orientation and field trip	D. Goehringer
19:30	Lab: Examination of salt marsh samples	(WHOI)
W 14 08:30 **	Deep sea hydrothermal vents	H. Jannasch
10:00	Anaerobic food webs: Fermentations	A. Salyers
14:00	Lab: Isolation of lactic acid bacteria, amino acid fermenters	
19:30	Presentations of research interests	Students
Th 15 08:30	Anaerobic food webs: Methanogens	Salyers
10:00	" " " Acetogens	"
14:00	" " " Sulfidogens	"
15:30	Lab: Isolation of anaerobic respiratory bacteria	
19:30	Microbial diversity: molecular analyses I	Doré
F 16 08:30	Discovery of oxygenic photosynthesis	Overmann
10:00	Anoxygenic phototrophy	"
14:00	Purple non-sulfur phototrophic bacteria	"
Sa 17 08:30	Starvation survival response in bacteria	D. Siegele
		(Texas A&M)
10:00	Microbial Diversity: Molecular analyses II	Doré
Su 18	The Beach? Whale-watching? Martha's Vineyard? Nantucket? Boston?	
M 19 08:30	Red sulfur phototrophic bacteria	Overmann
10:00	Green sulfur phototrophic bacteria	"
14:00	Lab: Isolation of phototrophic bacteria	

	19:30	...continued	
Tu 20	08:30	Cyanobacteria I	J. Waterbury (WHOI)
	10:00	Cyanobacteria II	"
	14:00	Cyanobacteria, lab	"
	19:30	...continued	
W 21	08:30	Chemolithotrophy	E. Leadbetter
	10:00	Bacterial motility I	"
	14:00	Microbial diversity: Molecular analyses III	J. Doré
Th 22	08:30	Phototaxis in phototrophic bacteria	C. Bauer
	10:00	... continued	(Indiana U)
	14:00	Phototaxis, laboratory	
F 23	08:30	Bacterial motility II	E. Leadbetter
	14:00	Phototaxis, laboratory	C. Bauer
Sa 24	08:00	** <b>MINI-SYMPOSIUM I: MICROBE-MACROBE INTERACTIONS</b> (see separate listing of speakers, events)	
S 25		Unscheduled	
M 26	08:30	Chemolithotrophy, revisited	E. Leadbetter
	10:00	Alternative cultivation techniques	A. Salyers
T 27	08:30	Starving bacteria, or What Monod didn't know	W. Chesbro
	10:00	.... continued	(Univ. New Hamp.)
	14:00	Lab	
W 28	08:30	In-situ hybridization procedures:	S. Nierzwicki-Bauer
		Development, use w/ cultured isolates	(R.P.I.)
	10:00	Use of probes w/ environmental samples	"
	14:00	Lab: Hybridization probes	"
Th 29	08:30	Geological, microbiological, hydrological	"
		Experiment: Case study w/ probes	"
	10:00	Laboratory, continuation	"
	14:00	" "	"
F 30	08:30	Discussion: probe methodologies, limitations	"
	10:00	Development of probes for project use	"
	14:00	Laboratory, continuation	
JULY:			
Sa 1	(07:15 bus departure for ferry trip from Portsmouth, N.H., to Isle of Shoals, to meet S.E.A. vessel for oceanic sampling)		
Su 2	(evening)	Return	
M 3	08:30	Overview of bioluminescence	P. Dunlap
	10:00	Physiology/molecular genetics	(WHOI)

	14:00	of bacterial luminescence Lab: Isolation of luminescent bacteria	"
T 4	14:00	Holiday: morning is free Laboratory: bacterial isolations	
W 5	08:30 10:00	TBA TBA	
Th 6	08:30	Tales from the crypts: development of a benign infection in bacteria-squid light organ symbioses	E. G. Ruby (Univ. So. Calif.)
F 7	08:30	Migration in mats	E. Leadbetter
Sa 8	08:00 14:00	** <b>MINI-SYMPOSIUM II : BACTERIAL ADAPTATION continued</b>	
Su 9		Unscheduled	
M 10	08:30 10:00 14:00 03:30	Gene transfer in natural habitats Development of new genetic systems Lab: Plasmid diversity and isolations Students' progress reports	A. Salyers " N. Shoemaker (U. of Ill.)
T 11	08:30	Work on individual projects, isolations	
W 12	08:30 10:00	Lactic acid producing bacteria Ciliates and other local protozoa	E. Leadbetter M. Sogin (MBL)
Th 13	08:30	Propionic acid bacteria, Clostridia, acetogens	E. Leadbetter
F 14	08:30	Bacterial and Archaeal hyperthermo- philes: comparing apples with apples	K. Noll (Univ. of Conn.)
Sa 15	08:00	<b>MINI-SYMPOSIUM III : BACTERIAL ADAPTATION II</b>	
Su 16		Unscheduled	
M 17 - T 18		No lectures scheduled; research project time	
W 19	08:30	Pseudomonads and other aerobes	E. Leadbetter
Th 20	08:30	Marine clinical microbiology	B. Bullis (MBL)
F 21	08:30	Discussion of enrichment and isolation results	
Sa 22		No lectures scheduled; research project time	
Su 23		Unscheduled	
M 24		No lectures scheduled; last day to work on research projects	



Tu 25	08:30	TA reports on group enrichments/isolations; lab clean-up begins
W 26	02:00	Begin students' project presentations
Th 27	08:30	Continue project presentations
	10:30	" " "
	14:00	" " "
	20:00	"Commencement", social

## SCHEDULES FOR MINI-SYMPOSIA--1995

### Microbial Diversity Program Marine Biological Laboratory, Woods Hole MA

#### 24 June I. Microbe-Macrobe Interactions

- 8:00 a.m. Prof. Paul Baumann, Univ. of Calif., Davis  
Biology of Buchnera (symbionts of aphids)
- 9:20 a.m. Prof. Colleen Cavanaugh, Harvard Univ.  
Invertebrate-chemoautotroph symbioses: adaptation  
at oxic-anoxic interfaces
- 10:45 a.m. Prof. S. Ferrand, Univ. of Illinois  
Resource utilization fosters a plant-microbe interaction

#### 8 July II. Bacterial Adaptation I

- 8:00 a.m. Prof. Paul Blum, Univ. of Nebraska  
In situ bacterial growth state using protein biomarkers
- 9:20 a.m. Prof. Norman Pace, Indiana Univ.  
New perspectives on the natural microbial world: Molecular microbial ecology
- 10:45 a.m. Prof. A. N. Chakrabarty, U. Illinois-Chicago  
Molecular mechanisms and evolutionary insights in the  
microbial degradation of toxic chemicals
- 2:00 p.m. Dr. Alexander Zehnder, EAWAG, Switzerland  
Biological removal of phosphate from waste water

#### 15 July III. Bacterial Adaptation II.

- 8:00 a.m. Prof. Douglas Bartlett, Univ. Calif., San Diego  
Pressure sensing and pressure adaptation in the deep-sea bacterium *Photobacterium*  
SS9: Lessons of microbial unity and diversity
- 9:20 a.m. Prof. Susan Leschine, Univ. Mass., Amherst  
Cellulose-fermenting bacteria make the carbon cycle  
go 'round
- 10:45 a.m. Prof. Lily Young, Rutgers Univ.  
Diverse anaerobic processes and the metabolism of aromatic compounds

NOTE: Mini-symposia are held in Candlehouse 105. Speakers visit with  
students in the course laboratory, Loeb 208, Saturday afternoon.

## PROJECT REPORTS

A. Direct investigation of microbial diversity.....

A demonstration project conducted by Joel Dore, with student participation

B. List of student project titles

C. Student-prepared project reports (copies; many original reports contained photomicrographs in color; only black and white copies are provided here).

*Direct Investigation of Microbial Diversity  
of Environmental Samples  
Based on 16S rRNA cloning and Comparative Sequencing*

**MARINE BIOLOGICAL LABORATORY, Woods Hole  
Microbial Diversity Summer Course 1995**

Joël Doré

**A demonstration lab:**

The objective of the described laboratory was to introduce, as a satellite development, novel rDNA-comparative-sequencing based techniques to investigate the microbial diversity of complex communities.

The general outline is as described by Amann et al. (Microbiol. Rev. 1995, 59;143-169).

1. Samples collected during the first weeks of the course were subjected to "total" DNA extraction, using a procedure optimized for the extraction of intact genomic DNA from *Methanobrevibacter* sp. (Dore, unpublished).
2. The extracted DNAs were used for PCR amplification of the 16S rDNA genes using two sets of primers expected to selectively amplify the rDNA of Archaea (primer set A) or Bacteria (primer set B):

Forward primers (5'-3')

Primer set A	344F: ACGGGGCGCAGCAGGCGCGA
Primer set B	008F: AGAGTTTGATCCTGGCTCAG

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Common Reverse primer	1517R: ACGGCTACCTTGTTACGACTT
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3. The PCR products were ligated into the pCRII vector for transformation of *E. coli* cells using the Invitrogen TA-cloning system.
4. Clones were screened by RFLP of colony-PCR reamplified inserts (using the M13 primers).
5. Plasmids from clones of interest were extracted using the Quiagen purification columns
6. Inserts were sequenced downstream of the M13-Forward primer with readings of 100 to 500 bases. Sequencing was done in Mitchell Sogin's Laboratory.

**Questions raised...:**

The primary goal of this lab. was to assess the feasibility of the type of work undertaken in the setting of the summer course. Therefore, the essential point of the questions raised suffered limitations. Indeed it is questionable whether the technology described therein should be applied to an observational approach of microbial diversity when it can find a high degree of rationality if combined to (additive probes-) quantitative hybridization based molecular ecology studies, for instance. Ribosomal-RNA targeted hybridization probes available for ecological studies allow the comparison of universal and domain signals, or the study of phylogenetic groups and subgroups. The utilization of such approaches allows to point out discrepancies between expected additive signals based on the known diversity of cultured-characterized strains and the observed molecular diversity. Due to the lack of such basic observations, the work undertaken remained purely observational. Whenever applied to environmental samples, direct molecular investigations of microbial diversity have led to the observation of new, formerly uncharacterized, lines of descent. Yet the microbial communities studied to date were mainly those of extreme environments for which culture collections are limited. We herein had the opportunity to test the general aspect of this observation using gut samples as starting material, for which the microbial community is far better known, although likely not thoroughly. It should be noticed that the actual quantification of diversity would require the characterization of thousands of clones and sequence based analysis would in this case go beyond the capabilities of the course. Alternative methods may be implemented to this end.

**Removal of water-solubles:** (applied to samples of gut contents)

1. Distribute 20 g samples in Stomacher bags
2. Add 20 ml PBS (pH 6.8 prepared in distilled water boiled and cooled under N<sub>2</sub>).
3. Mix in Stomacher for 30 seconds, and filter through 2 layers of gauze under N<sub>2</sub>.
4. Repeat steps 2 and 3.
5. Wash retentate with 160 ml PBS.
6. Centrifuge filtrate 1,500xg, 4°C, 5 min.
7. Centrifuge supernatant 25,000xg, 4°C, 30 min.
8. Dispense pellet in 250mg aliquots, in sterile Sarstedt tubes
9. Treat at 65°C for 15 min and/or store at -20°C. Note: -4°C for use within a month.

Note : Secondary purification of DNA extracts was also successfully used with fresh-water sediments "total" DNA which was not amplifiable.

**PCR amplification of ribosomal DNA fragments from environmental DNA**  
(protocole, primer sets, purification of amplification products)

**PCR protocole:** the general protocole outlined below was used as a starting point. Each PCR run (primer set-DNA matrix-Taq polymerase-PCR machine combination) may require optimization and adjustments. The proposed protocole is hot-start PCR using Perkin Elmer products. The description below has worked well for 100µl and 50µl reactions. The ERICOMP *EasyCycler* was used, set with *In Tube* temperature probe-control.

1. Lower phase reaction mix: (in each sterile 0.5 ml PCR microtube)

10X PCR buffer II	2.5µl	or	1X final concn.
dGTP 10mM	1.0-2.0µl		100-200µM
dATP 10mM	1.0-2.0µl		100-200µM
dTTP 10mM	1.0-2.0µl		100-200µM
dCTP 10mM	1.0-2.0µl		100-200µM

Primer R (10-25µM)	2.0µl	0.2-1.0µM
Primer F (10-25µM)	2.0µl	0.2-1.0µM
MgCl <sub>2</sub> (25mM)	4.0-16.0µl	1-4mM

ddH<sub>2</sub>O to : 25µl

spinn 12000rpm, 30sec.

2. Add PCR Ampliwax gemm (one in each sterile 0.5 ml PCR microtube) and melt in heating block 80°C, 2min. Let solidify at room Tre.

3. Upper phase reaction mix: (in each sterile 0.5 ml PCR microtube, overlay)

ddH <sub>2</sub> O	57.0µl
10X PCR buffer	7.5µl
AmpliTaQ DNA Polymerase	0.5µl or 2.5 units
DNA extract (~25µg/ml)	10µl or 1.0µg

4. Amplification program:

Initial denaturation 94°C, 2 min.  
40 cycles of: denaturation 92°C, 30 sec; reannealing 48°C, 30sec.;  
polymerization 72°C, 45 sec.  
final polymerization of partial products 72°C, 15min.  
hold 4°C.

5. Trouble shooting and optimization:

We have used Perkin Elmer GeneAmp PCR process Beginner's guide, Apple Macintosh version running with Hypercard. PC versions also exist. It gives helpful hints in a user friendly manual, and allows optimization of the PCR program itself, when this is the problem..

6. a micro-method was designed for optimization (see below) and used as a starting point to test amplifiability of dilutions of initial environmental DNA extracts (1 - 10<sup>-5</sup>).



4. add 1µl glycogen, 2 volumes pure ethanol and 1/20 vol 8M LiCl<sub>2</sub>.
5. Precipitate 1h at -20°C, pellet (10min., 14000rpm) and wash once with 70% ethanol.
6. dry pellet 2 min in speed-vac and dissolve in 35-50µl TE 1X( TrisHCl 10mM, EDTA 1mM). Rotating 30min. at c.a. 37°C might help for rapid and homogeneous dissolution.

## **Cloning of PCR amplified-environmental rDNA (ligation, transformation, preparation of sequence-ready plasmids,...)**

### **TA Cloning.**

In most cases former methodologies called for the use of modified primer sequences containing the rDNA-complementary sequence and a stretch of added sequence containing specific restriction sites used for cloning in classical vectors.

We used the InVitroGen system for cloning PCR products. It has been recently applied by several laboratories for the cloning of rDNA amplicons and appeared appropriate for this purpose. The TA cloning system makes use of a characteristic of the PCR products which is due to a terminal transferase activity common to native thermophilic enzymes (from *T. aquaticus* and *T. flavus*): the possession of a single 3' deoxyadenylate residue to each end (Clark J.M., 1988. NAR 16:9677-9686). The TA cloning kit provides plasmid (pCR<sup>TM</sup>II), enzymes and competent *E.coli* cells and allows blue/white screening as well as Kanamycin or Ampicillin selection.

It was found highly relevant to test both the 1:1 and 3:1 ratios of insert to plasmid in the ligation for successful cloning using the Invitrogen kit.

### **preparation of sequence-ready plasmids.**

Obviously, an overwhelming number of procedures are available to achieve this goal. For automated sequencing, the quality of the template is of great concern. We used the **Quiagen Quia-Prep method** for *E. coli* plasmids mini-prep. Slight modifications of the manufacturers description included a spinning of the first cell lysate instead of filtration through QuiaFilters, and three washes of the bound plasmid DNA before elution.

### **Screening of clones (sizing, restriction digest, 5'-end sequencing,...)**

Primary screening is necessary when a large number of clones have been obtained and may represent numerous copies of each initial rDNA. We used restriction fragments length polymorphism analysis of the full plasmid or the re-amplified rDNA insert:

1. Sau3a which is a frequent cutter.
2. a combination of SmaI + Kpn I, which do not cut more than one site on the

plasmid itself, can also be used.

Alternatively or in combination, single base sequencing may be used as a rapid screening method to eliminate all duplicate (similar) clones.

The following is the procedure used to re-amplify rDNA inserts for size and restriction screening.

### **micro-PCR for rDNA-TA clones testing**

For (# reactions) :

	10	15	20	25
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1. transfer 5µl AmpliWax to PCR tubes working in thermocycler set at 80 degrees
2. dispense 5µl ddH<sub>2</sub>O to each tube
3. transfer to each a toothpick sample from an LB-Kanamycin-XGal grown white colony
4. denature at 95 degrees for 10 min and cool at room temperature
5. prepare reaction mix :

10X PCR Buffer	25.00
dNTPs 10mM each	2.50
M13F- primer (2pmol/µl)	5.00
M13R- primer (2µM)	5.00
MgCl <sub>2</sub>	15.00
ddH <sub>2</sub> O	100.00
AmpliTaq	2.50
total	150.00

### Alignment and phylogenetic positioning

The sequence was then transferred to the UNIX-based GDE software available free from the RDP and other sources. Many other softwares can be used to the same end.

Sequences were first aligned with a most similar one using the RDP database. Conserved regions evidenced by the Blastn search were aligned first and the more variable regions aligned from there. A mask was created to remove any gaps and regions of uncertain alignment from the phylogenetic analysis. Considering only partial sequences were obtained, the information is only valid in terms of evolutionary relationship at the level of the phylogenetic cluster.

## SUMMARY OF RESULTS:

### DNA extractions

All samples tested, including

- Sippewissett Marsh "red berries" aggregated forming at bottom of transient salt-water pools
  - termite gut contents
  - Fresh-water Cedar swamp sediments [Project of Christof Holiger]
  - Subsamples of a marine (Buzards bay) sediment core sample [Project of Costantino Vetriani]
  - The squid's accessory nidamental gland [Project of Chih-Ching Chien]
  - halophilic bacteria and archaea in pure culture [Project of David Arahal]
  - cyanobacteria [Project of Grazyna Sroga]
- yielded DNA extracts of good quality (mostly intact genomic DNA)

### rDNA PCR

The fresh-water sediment DNA from Cedar-swamp were highly "colored" from co-extracted humic acids and likely other aromatic compounds that appeared inhibitory to PCR-amplification. These were the only samples that remained refractory to PCR, even after a secondary purification using Pierce X-treme spin columns. The pre-washing in PBS was not attempted.

For all other samples a dilution allowed PCR amplification of bacterial rDNA giving, under the conditions described, a single major band of 1500bp.

Direct rDNA PCR was attempted on cells from cultures or enrichments (Halobacteria, *Chromatium*-like organisms,...) but failed to give PCR products.

### rDNA cloning, sequencing and phylogenetic placement

Attempts to clone rDNA-PCR products were made using "red berries", termite gut contents, marine sediments and squid gland derived rDNA pools. The TA-cloning was optimized to give a large number of clones from various PCR products from the marine sediment sample, although little success was obtained with the squid gland sample. A few clones initially obtained from the "red berries", termite gut and also from human fecal DNA used as an initial demonstration sample were fully characterized by sizing and screening. Part of these, containing an insert of the expected size were subjected to sequencing, which did not "yield" easily.

Results of partial sequencing allowed the very primary positioning indicated in the figures below.

#### Form salt marsh "Red berries":

8 clones were obtained, 4 clones were selected by primary screening and two were used for rDNA sequencing. Both sequences were chimeric with a split in sequence around position 90-100. This was confirmed by the Check\_Chimera function available with the RDP email server. The longest sequence stretch of sequence available for alignment and sequence comparison indicated a close evolutionary relationship with sulfate-reducing bacteria and based on sequences available in the RDP database, a clustering with *Desulfobulbus* sp. It would be interesting to verify the presence of these organisms using *in situ* hybridization based on available probes for the different groups of SRB. The limited substrate range of electron donors available for *Desulfobulbus* sp. also makes this observation interesting with reference to the overall functioning of the "red berries" microbial community.

#### From termite gut contents:

15 clones were obtained, 12 were selected and 4 were used for rDNA sequencing including one derived from the archaeal domain PCR-primer set. The latter was in fact representative of a eukariotic member of the termite gut microbiota. The sequence obtained was identical to that of a clone recently characterized in Mitchell Sogin's laboratory as being an early branching protist related to trichomonads. *In situ* hybridization is likely to be investigated in Mitchell Sogin's laboratory. The three eubacterial clones were all very different. Clone 4 was related to the Spirochetes and clearly stemmed off of the Treponema group, with *Spirochaeta stenostrepta* as its

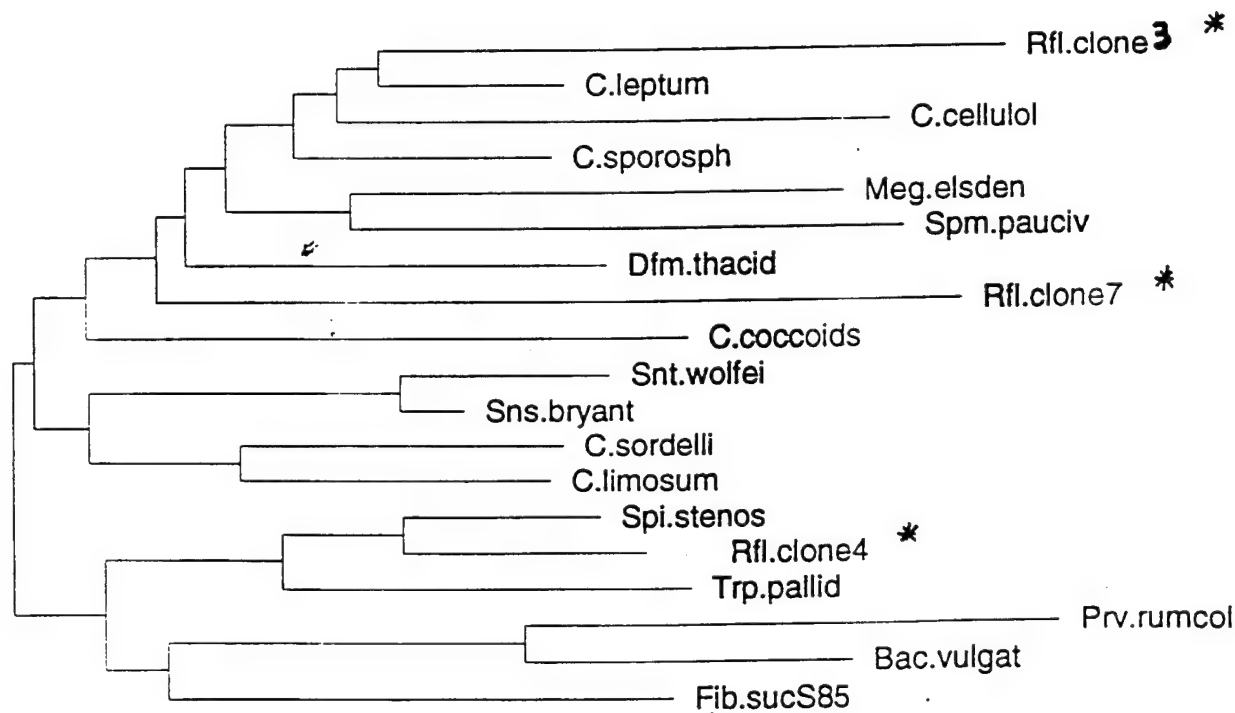
closest relative based on the RDP database. Clone 3 appeared to branch within the *Clostridium leptum* group with cellulolytic clostridia as close evolutionary relatives. Clone 7 was also found to be a chimeric sequence. Its longest stretch of homologous sequence (300 bp) appeared as deeply branching within the Clostridial assemblage. The latter would clearly need further sequencing to clarify its evolutionary positioning.

Other clones sequenced included 4 from human fecal DNA and over 10 from the marine sediment core sample. The latter were sequenced too late in the course for the analysis to be presented here.

#### Suggested Improvements would include:

1. *Addressing questions relevant to microbial ecology or uncultured microorganisms.* The Microbial Diversity Course appears as an ideal setting for the unambiguous phylogenetic placement of yet uncultured microorganisms. Furthermore, the development of hybridization-based techniques should allow (should it be over several years) to identify microorganisms detected but absent from culture collections. This approach offers the advantage of focussing on dominants/active organisms from the start. On the other hand, the rDNA based sequencing studies yielding information on clones such as the *Desulfobulbus*-related clones from the "red berries" or the *Treponema*-related clones from the termite gut could "feed in" future studies of *in situ* hybridization-based identification and quantification of the organisms.

2. *Practical elements: setting of a PCR laboratory with its specific equipment, and harmonizing the relation with the main lab.* The 1995 experience allowed to solve a number of practical difficulties inherent to setting PCR technology in an "unwilling" environment. It seems essential that the PCR venture be kept as an island to help with keeping a favorable working environment for PCR and prevent dissemination of "nightmare"-contaminants.



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\*. *Reticulosphaerula planipes* gut. rDNA clones.

## STUDENT PROJECTS

ANDERSON, RON

"Studies of Hind-Gut Microbiota of Termites"

ARAHAL, DAVID R.

"Characterization of Some Moderate and Extreme Halophiles from Culture Collection Strains and Environmental Samples by Comparative rRNA Sequence Analysis"

CHIEN, CHIH-CHING

"A Study of the Diversity of Microbiota in the Accessory Nidamental Gland of Squid *Loligo pealei*"

DAMGAARD, LARS RIIS

(1) "Microbial Transformation of Sulfide and 3-mercaptopropionate"

(2) "Agar Degrading Bacteria - A Small Study"

GEORGE, ALISON

"Microbial Reduction of Phosphate?"

HE, JIANCAI

"Isolation of Cellulolytic Bacterium using Turnip as Cellulose Source (Substrate)"

HILARIO, ELENA

(1) "Partial Stratification *In Vitro* of Microbial Subpopulations from the Salt Marsh Microbial Mat, Sippewissett MA"

(2) "Bacteriophage Extract from the Microbial Mat from Sippewissett MA"

HOLLIGER, CHRISTOF

"On the Use of Molecular Techniques for the Characterization of Methanogenic and Sulfate-Reducing Enrichments"

HOSKINS, DIONNE

(1) "Cultivation of Anaerobic Bacteria from the Human Vagina"

(2) "Growth and Amine Production by Anaerobic Bacteria in the Human Vagina"

MILLER, ERIC S.

"Survey and Isolation of Bacteriophages of Photosynthetic Purple Non-Sulfur and Marine Luminous Bacteria"

NEWMAN, DIANNE

"Tracing the Development of Natural Biofilms with *In Situ* Hybridization and Scanning Confocal Laser Microscopy"

PLUGGE, CAROLINE

(1) "Enrichment of *Chlorochromatium aggregatum*"

(2) "Degradation of Methanesulfonate by Aerobic Bacteria"

SABL, JOY

"Use of Canada Balsam as a Selective Agent and Possible Substrate in the Enrichment of Termite Hindgut Microbiota"

SCHLEKAT, CHRISTIAN E.

"Characterization of the Microbial Community Associated with the Shipworm, *Lyrodus pedicellatus*"

SROGA, GRAZYNA E.

"The Physiology of N<sub>2</sub> Fixation by Non-Heterocystous Cyanobacteria"

SUAREZ-SANCHEZ, PAULA

"Taurine Utilization by Purple Non-Sulfur Bacteria"

VETRIANI, COSTANTINO

"Microbial Diversity in Anoxic Marine Sediments"

ZANNONI, DAVIDE

"The Effect of Respiration on the Phototactic Behaviour of the Purple Non-Sulfur Bacterium *Rhodospirillum centenum*"

## Enrichment and Isolation of Methanogens from Termite Hindgut

by Ron Anderson

### Introduction

Termites are estimated to produce between 25 and 150 Tg/year of methane (Hackstein and Stumm, 1991; Kane and Breznak, 1991). They are a small but an important source of methane in the atmosphere, and they make a significant contribution to global warming.

Methane is produced by methanogens located in the termite hindgut (Breznak and Brune, 1994). Methanogens are joined by a consortium of cellulolytic protozoa and other bacteria to process wood and other materials ingested by the termite. The ingested materials are degraded into low molecular weight molecules that are used by the termite for nutrition. Protozoans provide the enzymes needed to degrade the cellulose into glucose. Glucose is then fermented to produce acetate. Acetate is the major carbon source for the termite.  $\text{CO}_2$  and  $\text{H}_2$  are also produced during the degradation of the cellulose to acetate.

Methanogens use  $\text{CO}_2$  and  $\text{H}_2$  to form methane.  $\text{H}_2$  is provided to the methanogen by the protozoa by a process known as intra-species  $\text{H}_2$  transfer (Brauman *et al.*, 1992; Breznak and Blum, 1991). Acetogens also use  $\text{CO}_2$  and  $\text{H}_2$  to produce acetate. They are in competition with the methanogens for these materials. In most situations (such as the cow rumen), methanogens out-compete the acetogens. However, in the termite gut, acetogens utilize most of the  $\text{CO}_2$  and  $\text{H}_2$  to produce acetate which is used by the termite for nutrition. The methane produced by the methanogens from the remaining  $\text{CO}_2$  and  $\text{H}_2$  is expelled into the atmosphere.

Methanogens require stringent anaerobic conditions for growth and are probably restricted to the

parts of the hindgut that are anaerobic (Bignell and Anderson, 1980; Brune *et al.*, 1995). Analysis of the hindgut by microelectrode probes has shown that there is an anaerobic zone in the shape of a tube down the center of the gut. Since the cells in the gut wall are oxygenated, the anaerobic zone is probably protected from O<sub>2</sub> by a mass of poorly mixed O<sub>2</sub> utilizing material next to the gut wall.

The epithelial cells in the hindgut are mucus free (Bignell *et al.*, 1980). Mucus would normally be expected to protect the gut wall from abrasion. Instead, spines are located in the gut wall to protect the gut wall and to assist the movement of material in the hindgut. There are structures on the outside of the gut wall that support and move the spines. The spines may be hollow and transport nutrients out of the hindgut (Bignell *et al.*, 1980).

The reactions to reduce CO<sub>2</sub> requires a unique cofactor know as F<sub>420</sub> (Cheeseman *et al.*, 1972). F<sub>420</sub> is autofluorescent. It has an absorption peak at 420 nm and fluoresces with a blue-green light. Methanogens can be located in the hindgut by observation of the blue-green light using a UV microscope (Doddema and Vogels, 1978).

Methanogens can be rods, cocci, or filaments (Breznak and Pankratz, 1977; Brune *et al.*, 1994). Their location in the gut is unknown. There are several likely locations for the methanogens in the hindgut. They may be free swimming in the gut contents. They may be intra-cellular symbionts in some of the protozoa (Gilzen *et al.*, 1991; Lee *et al.*, 1987; Odelson and Breznak, 1985; To *et al.*, 1980). They may coat the surface of filamentous bacteria located in the gut. They may be located in special tissues. Also, they may be attached to the spines from the gut wall (Breznak and Pankratz, 1977).

There are ways to stimulate methane production and promote the growth of methanogens in the

termite gut. The production of methane by methanogens can be increased by incubating the termites in an atmosphere of  $H_2$  and  $CO_2$  (Messer *et al.*, 1989). The growth rate of the methanogens also probably increases. In cockroaches the production of methane and the growth rate of the methanogens can probably be increased by the diet of the termite (Kaesler and Schonheit, 1989). This may also be true in termites. Feeding termites a special diet and incubating them in a  $CO_2$  and  $H_2$  atmosphere should also increase the density of methanogens and make isolation of methanogens easier. New methanogens may be found in the termite hindgut by this method.

The objective of this project is to isolate methanogens by an *in vivo* enrichment. A feeding study will be conducted to screen various materials for their effect on the methanogen population and methane production. The termites will be fed a diet that increases the density of methanogens in the hindgut and will be incubated in a  $H_2/CO_2$  atmosphere to stimulate the growth of methanogens and methane production. Normal enrichment procedures will be followed to isolate methanogens from the enriched termite gut. It is hoped that this approach will allow the isolation of methanogen that have not been isolated previously. It is also expected that the location of the methanogens in the gut can be determined by microscopy. Any methanogens isolated by this procedure will be further characterized.

### Materials and Methods

**Termites** - Specimens of the lower termite species *Reticulitermes flavipes* were collected from cardboard scraps placed under large pieces of wood in E. Leadbetter's back yard. The termites were trapped on the outer surface and in the folds of the moist cardboard scraps. Termites were collected and trapped in large groups within 24 hours of setting the cardboard traps. The termites were maintained for up to four days in the laboratory on moist cardboard and soil. Both



soldier and worker termites were collected in the traps. Only the worker termites were used for experimentation.

**Enrichment Media** - The enrichment medium was the anaerobic medium (AC-21) described by Breznak *et al.* (1988) modified by using the trace elements described by Greening and Leedle (1989). The medium was made and dispensed by Hungate procedures for stringent anaerobes. The antibiotics Cephalotine (20µg/ml of medium) and Clindamycine (4µg/ml of medium) were added to the medium in some experiments. In some experiments bottle plates were used. The medium was AC-21 medium with agar at a final concentration of 20g/l. In all experiments the inoculum was mixed or slurred in enough bovine rumen fluid to give a final rumen fluid concentration of 10%. The rumen fluid was filtered, centrifuged and autoclaved before use. In all cases the shake tubes or bottle plates were gassed with a H<sub>2</sub>/CO<sub>2</sub> mixture (80/20 v/v) to a 30 psi. Nutrient agar was used to plate aerobic spore forming bacteria. All samples were incubated at room temperature in the dark.

**Feeding Experiments** - Groups of up to 15 termites were placed in divided petri dishes and stored in the dark for one or two days. The food source was a drop of solution placed at the intersection of the divider and the petri dish wall. The food sources were xylan, D-xylose, D-cellobiose, chitin, potato starch, sodium formate, sodium acetate, citric pectin and dextrose. They were used with enough water to form clear solutions or pastes (25% to 50%). Some termites were fed antibiotics by wetting Whatman filter paper with dilute solutions of Cephalotine and Clindamycine. Termites for control experiments were fed moist Whatman filter paper and stored in petri dishes. After the feeding phase the termites were transferred to 10 ml GC sampling vials with a septum closure and flushed with H<sub>2</sub>/CO<sub>2</sub> (80/20 v/v) for 15 seconds. After storage at room temperature the head space in the vials was sampled for analysis and the termites were removed for microscopic analysis.

**Microscopy** - The bacteria in the termite hindgut were observed by both phase contrast and fluorescence microscopy. A Zeiss microscope with phase contrast optics and a UV light source was utilized for both microscopic techniques. The microscope was capable of rapidly changing between the two methods. F<sub>420</sub> was observed by illuminating the sample with UV light at 400 to 420 nm. The sample was observed through a low pass filter at 470 nm. It was noted that the blue color emitted by F<sub>420</sub> would fade after a few minutes of exposure to UV light. It is known that F<sub>420</sub> must be in oxidized state to fluoresce (Cheeseman *et al.*, 1972; Doddema and Vogels, 1978). For some observations an oxidation pretreatment was conducted by mixing the sample on the slide with a few crystals of potassium ferricyanide. This assured that the F<sub>420</sub> was oxidized and enhanced the capability of observing the methanogens.

A single evaluation was made using the Nikon confocal microscope.

**Head Space Gas Analysis** - The methane in the head space of enrichment cultures and termite feeding studies was determined by gas chromatography using a Shimadzu GC-14A gas chromatograph with a 6' x 1/8" Supelco 80/100 Parapak R column and a flame ionization detector. The column temperature was 55°C. The injector temperature and the detector temperatures were 80°C. The sample injection volume was 40 µl. The carrier gas was helium, and the carrier gas inlet pressure was 1 kg/ cm<sup>2</sup>. The GC method was calibrated with samples of known methane content. The data was recorded and analyzed using a Shimadzu CR501 integrator.

**Enrichment Techniques** - Bottle plates were used to provide a solid surface for the growth of methanogens and other hindgut bacteria. The bottle plates were inoculated with enough culture to cover the medium with culture to a depth of approximately 20 mm.

**Microelectrodes** - Microelectrodes were used to determine the pH, methane concentration, and the O<sub>2</sub> concentration in the hindgut. The microelectrodes (described by Revsbech *et al.*, 1986) were provided by L. Damgaard. The experiments were similar to the measurements described by Brune *et al.* (1995). Live termites were imbedded in 2% agarose strips formed between two glass microscope slides. To protect the microelectrodes during measurements the agarose strip was removed from the glass slides and positioned on the surface of agar medium in a plastic petri dish. The electrode was mounted on a computerized positioning device capable of accurately positioning the microelectrodes. The microelectrode was positioned to first penetrate the outer structure of the termite and then the hind gut. Measurements were made every 0.1 mm until the microelectrode penetrated the far side of the termite.

## **Results**

**Microscopic Analysis** - The hindgut from the termite was removed by pulling the end of tail away from the midsection with tweezers. Usually the hindgut would separate from the stomach intact and was positioned in a straight line on a glass slide. The hindgut was then opened by dissection with a razor blade or split open by rubbing the intact hindgut between a glass slide and a cover slip. Generally the best slides were prepared by rubbing the hindgut between the slide and the cover glass. A wet mount was made using a drop of anaerobic medium. The contents of the hindgut were then observed microscopically.

The wet mounts were first observed by phase contrast microscopy. The hindgut contents consisted of bacteria, protozoans, gut wall structures, and liquid. The liquid material was of unknown composition, but it contained cellulosic matter ingested by the termite during feeding. The wide range of microorganisms including spirochetes, filamentous bacteria and cocci bacteria,

and many unidentified protozoans. The protozoans and spirochetes showed rapid motility in fresh wet mounts. After several minutes the motility would cease beginning with the microorganisms located at the edges of the wet mount. Presumably oxygen diffused into the sample from the sides and the microorganisms were killed. The high density of protozoans and other unknown materials in the hind gut made recognition of bacteria impossible. The structures of the hindgut wall were observed, and the spines attached to the gut wall were identified. Identification of other specific structures was not possible.

The microscope light source was switched from visible light to UV light with a wave length of 420 nm. The wet mounts were then observed to detect fluorescent materials. Many fluorescent materials were seen but the blue green and yellow materials were the overwhelming majority. The blue green structures were either long filamentous materials associated with the gut wall, free swimming cocci, or nonuniform materials located inside the protozoans. Figure 1 shows some of the blue green structures that were observed. The yellow materials were nonuniform blobs located either free in the hindgut liquid or inside the protozoans.

To assist interpretation of the UV microscope observations, fresh pine sawdust and cellulose fibers (Whatman filter paper) were observed. Cellulose (Whatman filter paper) did not fluoresce. Fresh saw dust from pine board fluoresced only with a yellow color similar to the color observed in the saw dust. It is assumed that the aromatic components of the lignin is the material detected by the yellow color. It was concluded that the blue green materials were methanogens.

The blue green color faded after several minutes exposure to the light source. The fading was thought to be due to changes in oxidation state of the F<sub>420</sub> cofactor. Some wet mounts were pre-treated with oxidizing agent to enhance the blue green color before UV observation.

The hind gut was extensively observed. Many wet mount views were observed by both phase contrast and UV techniques by switching rapidly between the two techniques. The locations of methanogens in the mass were determined by comparing the phase contrast and UV views. Slides and a video tape were made to record the results (not included here).

A single hindgut sample was observed using confocal microscopy. The light source was a laser with an adjustable wave length (420 nm). The result of the analysis is shown in the black and white picture in Figure 2. The filamentous structures are thought to be methanogens. It should be possible to determine the color of the fluorescent material using a color camera. However, the correct combination of laser light source and camera was not available. The method was promising. I may be able to locate the methanogens attached to the hindgut structures.

**Methane Generation by Groups of Termites** - The production of methane by groups of termites was determined by storing groups of three termites in vials and measuring methane in the head space of the vial after 2 to 24 hours incubation (Table 1). In some samples the head space was initially exchanged with  $N_2/CO_2$  or  $H_2/CO_2$  gas mixtures. The generation of methane was unaffected by  $N_2/CO_2$  gas. The generation of methane was enhanced by exchanging the head space with a  $H_2/CO_2$  gas mixture. The survival rate of the termites was decreased by the gas exchange of both gas mixtures.

**Feeding Study** - A feeding study was carried out on groups of termites to determine the effects of diet on the microorganisms in the hindgut and on the generation of methane by methanogens. The feeding study consisted of two stages. During the first stage groups of termites were fed one of several food sources for 24 hours (Table 2). During the second stage subsets of termites from stage 1 were stored in 5 ml vials and the methane content of the head space was determined. At each stage termites were dissected and the hind gut was sampled and observed.

The results of the feeding study are seen in Table 2. Termites fed cellobiose, dextrose, sodium acetate, and sodium formate died during stage 1 and were discarded. The termites fed sodium acetate and sodium formate died after ingesting the materials. Termites fed cellobiose and dextrose died by sticking to the food source. Little of these materials was ingested by the termites. Microscopic observation during stage 1 showed the number of protozoans reduced during the feeding stage.

Termites from stage 1 were sampled and tested for methane generation (Figure 2). Methane production was lower for each food source except xylose when compared to the filter paper food source or filter paper impregnated with antibiotics. For some samples the head space gas was exchanged with  $H_2/CO_2$ . In each case methane generation was increased by the exchange of head space gas. Except for the xylan food source, the combination of food source and the exchange of head space gas resulted in increased methane production.

Microscopic analysis of the termite hind guts showed that all of the protozoans were killed with the exchange of head space gasses. Motile spirochetes remained in the hind gut liquid. The amount of blue green material was greater either because there were more methanogenic bacteria or the protozoans were not blocking the observation. Slide and a video tape were prepared to document the changes in the hindgut contents.

Termite survival rates were noted during each stage of the feeding study. Few termites survived the combination of feeding and head space gas exchange (Figure 2).

These observations indicated that the microbiota in the hindgut were significantly influenced by diet and  $H_2$ . The protozoans died. The microscopic observations suggests that the methanogens

were enriched in the feeding and  $H_2/CO_2$  treatments.

**Enrichment and Isolation** - Enrichments to assist in isolation of pure cultures of methanogens was attempted (Table 3). Anaerobic enrichments were prepared from the hindgut of both fresh live termites and live termites enriched for methanogens in the feeding study. Enrichment cultures were prepared in CA-1 medium in Hungate tubes followed by multiple transfer in tubes and bottles. The tubes were gassed with  $H_2/CO_2$  to 30 psig. The cultures were observed daily by UV microscopy to detect fluorescent methanogens and were tested daily for methane generation in the head space. Filamentous fluorescent bacteria were observed in the first culture and methane was detected in the head space. Samples were transferred to new tubes. Bottle plates were used in subsequent transfers to provide a solid surface for the growth of the filamentous bacteria. Methanogens did not grow rapidly in the subsequent transfers. While anaerobic filamentous bacteria were seen, no pure cultures were obtained.

Aerobic spore formers were found in the anaerobically enrichments. The enriched material was treated by centrifugation and pasteurization to isolate the filamentous and spore forming bacteria. Sample of both materials were streaked on nutrient agar. A pure culture of a bacillus bacteria was found from the pasteurization. Several red colonies were found on the samples from the centrifugation treatment. The colonies were restreaked and a pure culture of red rods was obtained.

**Microprobe Analysis** - Microprobes for pH,  $CH_4$ , and  $O_2$  were used to evaluate the conditions inside the termite hindgut. Each probe was inserted into the hindgut of live termites by penetrating the outside of hindgut. The pH probe showed that the interior of the gut was uniformly at pH 7. Methane levels in the hindgut were less than the detection limit of the probe. The  $O_2$  probe gave the best results (Figure 3). The analysis was repeated over several termites

and consistent results were obtained for each specimen. Stringent anaerobic conditions were found immediately after penetrating the gut wall and were maintained until the probe penetrate the other side of the hindgut. The extent of anaerobic conditions seemed to be greater than previously reported (Brune *et al.*, 1995).

### **Conclusions**

Based on this work the following is concluded:

1. Methanogens can be observed and identified in termite hindgut by a combination of microscopic techniques including confocal, UV, and phase contrast..
2. Methanogens in live termites can be enriched by diet, antibiotics and incubation in H<sub>2</sub> rich atmospheric conditions.
3. Methanogens are associated with hindgut tissues and structures.
4. Most of the termite hindgut is anaerobic. Only a small annulus at the hindgut is aerobic.
5. No pure cultures of methanogens were isolated during the summer project. Pure cultures of aerobic spore forming and filamentous bacteria were isolated from the hind gut by streaking enriched cultures on nutrient agar.

### **Recommendations**

The following projects are recommended for future courses:

1. Isolate spirochetes from the termite hindgut.
2. Isolate spore forming filamentous bacteria from the termite hindgut.
3. Isolate methanogens from the termite hindgut using *in vivo* enrichment techniques as described in this report.
4. Locate filamentous methanogens associated with the hindgut wall or spines using confocal microscopy.



**Table 1.** Methane Generation by Isolated Termites  
(methane as a percentage of the head space gas)

Atmosphere in Head Space (Ambient Pressure)	Methane Generation after 2 hr	Methane Generation after 4 hr	Methane Generation after 24 hr	Survival after 24 hr (live/total)
Ambient Air	0.02%	0.04%	0.15%	3/3
N <sub>2</sub> /CO <sub>2</sub> (80/20)	0.02%	0.04%	0.07%	1/3
H <sub>2</sub> /CO <sub>2</sub> (80/20)	0.08%	0.15%	0.56%	0/3

Note: Three worker termites were isolated in 5 ml sample vials with septum closures. The head space was exchanged with the gas mixtures noted and the vials were stored at room temperature in the dark.

**Table 2. Termite Feeding Study**  
(Methane produced by termites vs. termites fed paper only)

Food Source	Methane Generation after 24 hr Feeding	Methane Generation after 24 hr Feeding and H <sub>2</sub> Stimulation	Microscopic Analysis after Feeding Stage	Survival after 24 hr (live/total) In Air H <sub>2</sub>	
Whatman Filter Paper	100%	100%	No Protozoans	2/3	2/3
Whatman Filter Paper with Antibiotics	98%	92%	Some Protozoans	3/3	2/3
Cellobiose	All termites dead	after feeding stage.	-	Not tested.	
Cellulose	73%	138%	-	3/3	1/3
Chitin	57%	147%	-	3/3	1/3
Dextrose	All termites dead	after feeding stage.	-	Not tested.	
Pectin (Citrus)	38%	173%	Some Protozoans	1/3	1/3
Potato Starch	39%	138%	Some Protozoans	1/3	1/3
Sodium Acetate	All termites dead	after feeding stage.	-	Not tested.	
Sodium Formate	All termites dead	after feeding stage.	-	Not Tested	
D-Xylan	62%	103%	Some Protozoans	3/3	1/3
Xylose	116%	127%	No Protozoans	3/3	1/3

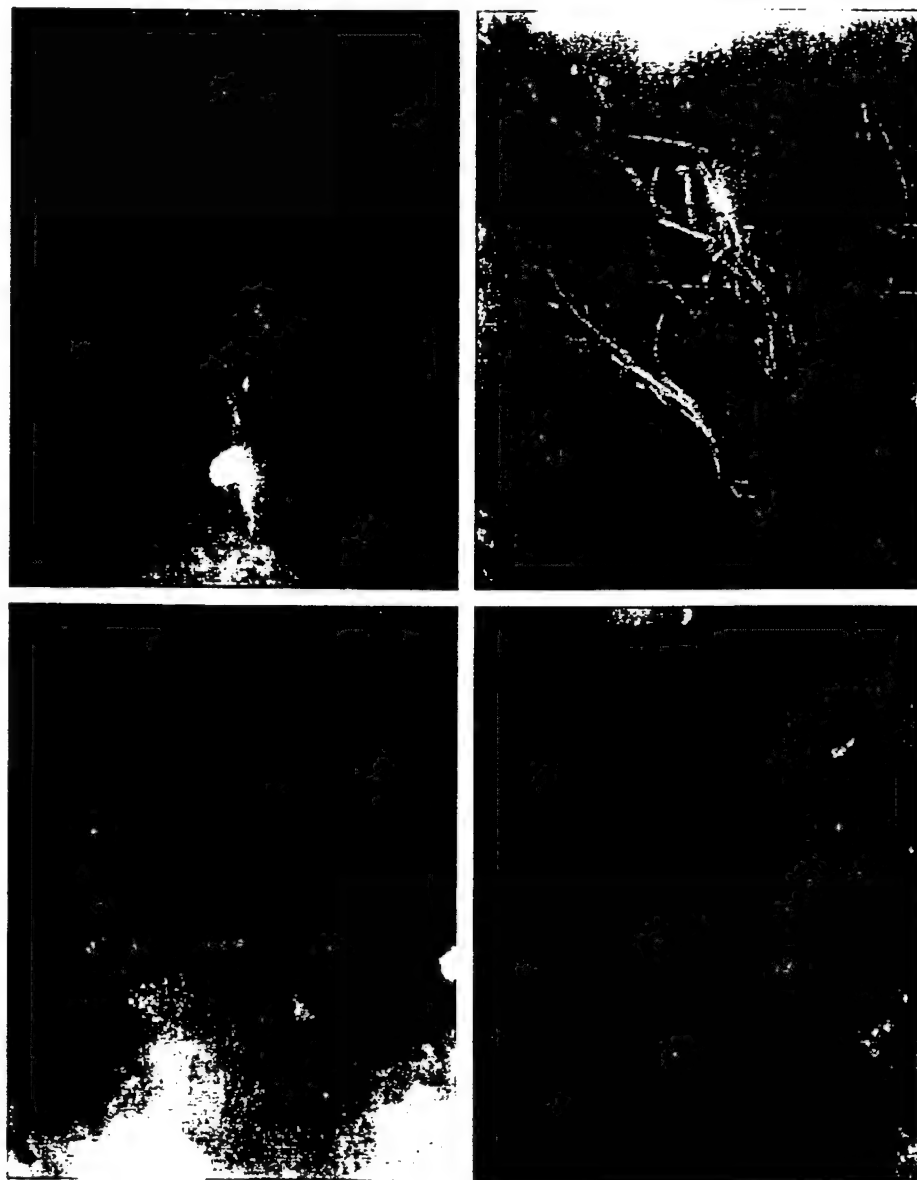
Test Procedure: Fifteen worker termites were isolated and fed the food source indicated above for 24 hours. After feeding, three termites were transferred to 5 ml vials and stored for 24 hours at room temperature. The amount of methane generated in the head space was determined by GC.

**Table 3. Enrichment for Methanogens**  
(anaerobic enrichment in Hungate tubes)

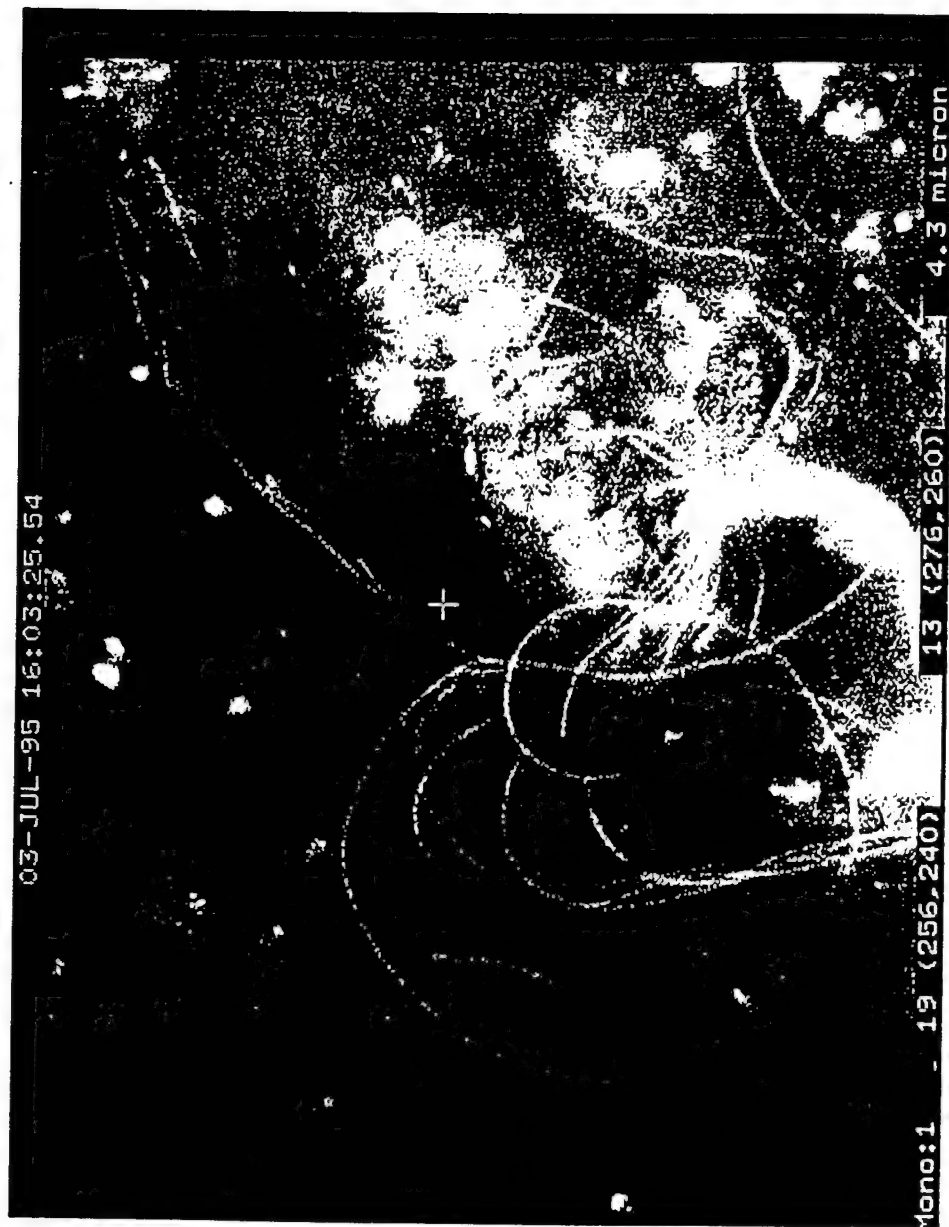
Inoculum	Antibiotics	Rumen Fluid	Methane Generation after 4 Days	Methane Generation after 14 Days	Visual and Microscopic Observations
Fresh Gut	Yes	Yes	0.02%	0.26%	Slight turbidity - No Methanogens Present
Fresh Gut	No	Yes	0.01%	0.15%	Slight turbidity
Fresh Gut	Yes	No	0.08%	6.62%	Cloudy with Methanogens Present
Fresh Gut	No	No	<0.01%	2.05%	Cloudy with Methanogens Present
Old Gut (with pectin)	Yes	Yes	0.03%	0.67%	Slight turbidity Methanogens present

Methane concentrations reported as % of head space gas above the base Methane generation of termites fed paper.

Test Procedure: Anaerobic tubes were inoculated with hindgut material slurred in anoxic medium. The head space was exchanged with  $H_2/CO_2$  (v/v) gas to 3 atmospheres.



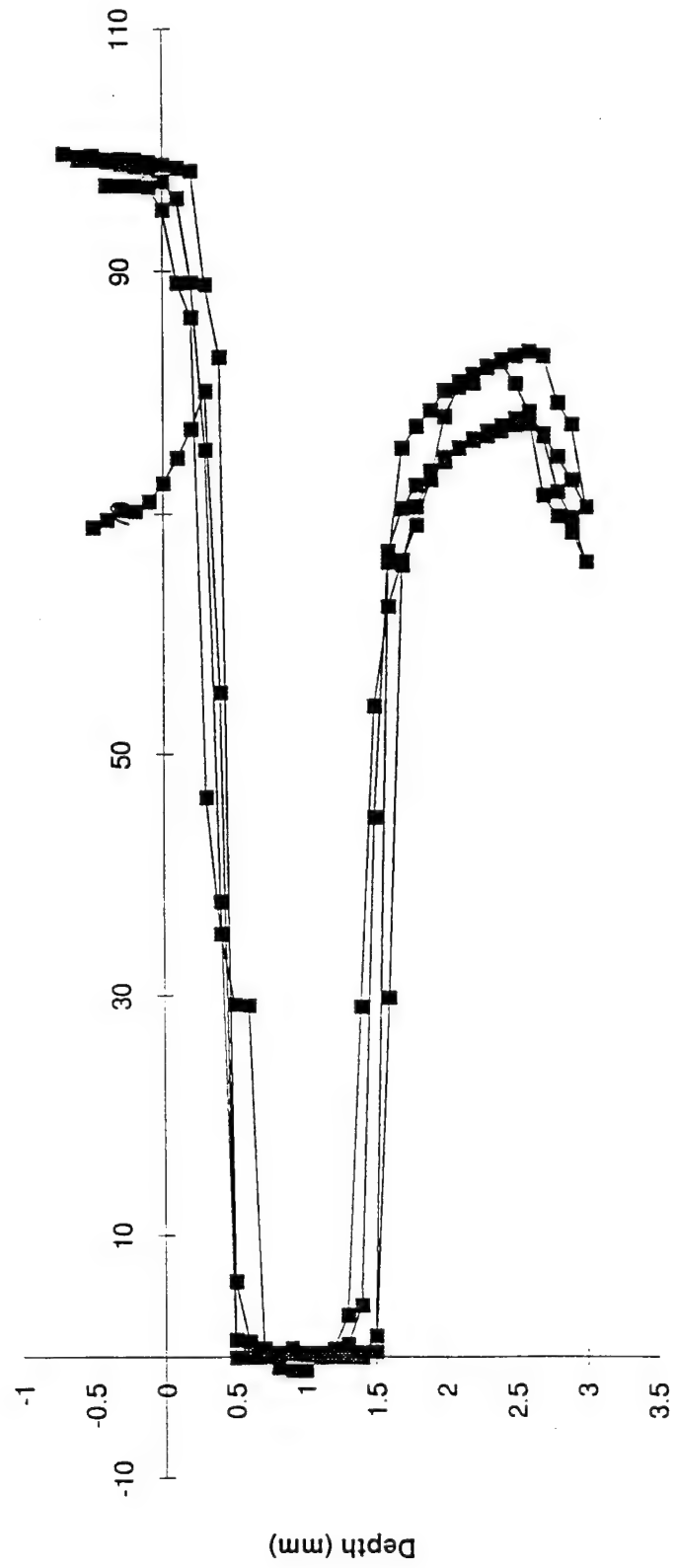
**Figure 1.** Photomicrographs of Methanogens in the Termite Hindgut  
(UV light source at 420 nm)



**Figure 2.** Confocal Photomicrograph of Methanogens in the Termite Hindgut  
(UV light source at 420 nm)

Figure 3. Oxygen Tension in the Termite Hindgut

Oxygen concentration as % saturation of air.



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# CHARACTERIZATION OF SOME MODERATE AND EXTREME HALOPHILES FROM CULTURE COLLECTION STRAINS AND ENVIRONMENTAL SAMPLES BY COMPARATIVE rRNA SEQUENCE ANALYSIS.

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## INTRODUCTION

rRNA and rDNA analysis is a powerful tool for characterization of microorganisms and the establishment of phylogenetic relationships. Especially interesting is the fact that environmental samples can be used for these purposes (1) as well as cell enrichments and of course pure cultures.

Since the pioneering studies of C.R. Woese in the 1960's, when he applied rRNA to comparative sequencing based evolutionary studies, a lot of work has been done and now rRNAs are accepted as ideal semantides (semantophoric molecules, i.e. genes or transcripts of genes bearing historical records of evolution). As a result of all this work a number of rRNA/rDNA databases are available for getting general information, submitting and retrieving sequences, etc.

In the case of moderate halophiles, defined as those microorganisms that are able to grow optimally in media containing between 3 and 15% NaCl (2), more

molecular analysis are needed in order to clarify the phylogenetic relationship of some of their representatives.

The extreme halophiles, defined as those microorganisms that grow best at 15 to 30% NaCl (2), can be considered a very well defined group, but yet rRNA analysis remains important to define their phylogenetic relationship to other groups and to make easier the identification and characterization of new isolates.

The strains that will be used for this project include four moderate halophiles: *Micrococcus halophilus* DSM 20408, *Marinococcus halobius* ATCC 21727, *Halomonas elongata*, *Hm. meridiana*; and eight extreme halophiles: *Halobacterium salinarium* CCM 2148, *Haloarcula vallismortis* ATCC 29715, *Ha. hispanica* ATCC 33960, *Ha. marimortui* ATCC 43049, *Haloferax volcanii* NCMB 2012, *Halococcus morrhuae* CCM 537, strain no. 617 and strain no. 704.

The environmental samples for this project were collected at different points of a large salt pile located at New Hampshire's port and consist of salt rocks of various types as well as a sludge from the margins of the salt pile. Also sea water (25 and 35 m depth) and sediment taken at Cape Cod Bay will be used.

## MATERIAL AND METHODS

Strains and culture conditions: The four moderately halophilic strains and the eight extreme halophilic strains used in this project are listed in the introduction section. All strains were grown in a medium containing 0.5% (w/v) yeast extract (Difco) in a salt mixture with a final concentration of 10% for the moderate halophiles and 25% for the extreme halophiles (4). The pH was adjusted to 7.5 and the incubation was at 37 C in an orbital shaker at 150 strokes per minute. When necessary, solid media was prepared by adding 2% (w/v) Bacto-Agar (Difco).

For the enrichments and isolations from the environmental samples both kind of media were used.

Isolation of genomic DNA and 16S rRNA sequence analysis: Cells were harvested at approximately late-exponential phase by centrifugation and resuspended in 200 to 400  $\mu$ L TE buffer (10mM Tris-HCl, 1mM EDTA pH 8.0). The cell lysis was accomplished by the addition of 200  $\mu$ L lysozyme (50 mg/mL), 20  $\mu$ L pronase (10 mg/mL), 8  $\mu$ L mutanolysine (5000 U/mL), 4  $\mu$ L RNAase (10 mg/mL) and incubation for 1hr at 37 C and gentle revolving motion. DNA extraction and precipitation were performed by the DNA extraction protocol by A. Sghir and J. Dore (unpublished).

Micro polymerase chain reaction (Micro-PCR) amplification of the 16S rRNA gene using the forward primer 008F and the reverse primer 1517R, as well as the forward primer 007F and the reverse primer 1517R, for rDNA-PCR optimization, was carried out with the DNA extracts. Where positive results were obtained the large scale PCR was performed.

The next steps include purification of the amplification products using Costar SpinX microcentrifugation filtration devices, cloning of PCR amplified-environmental and pure cultures rDNA (by either ligation, transformation or preparation of sequence-ready plasmids), screening of clones, sequencing, and phylogenetic positioning of environmental clones. At this point, dendrograms can be generated using a pairwise, weighted, least-squares distance method (3).

## RESULTS AND DISCUSSION

According to the methodology proposed the strains and samples were cultivated in liquid media, then the cells were harvested and their DNA was extracted following the procedures. These DNA extracts were checked by electrophoresis in agarose (1%) gel and no sign of contamination or degradation of the DNA was observed.

The dilutions (1:1000) of these DNA extracts were used for the DNA amplification and different results were obtained depending on the set of primers used. These data are shown in Table 1.

As it was expected PCR products using 008F-15171R primers were obtained only when DNA belonging to eubacteria was used. On the contrary only archaeobacterial DNA gave PCR products if 007F-1517R primers were used, since 007F is an specific primer for archaeobacteria. The sizes of the amplicates, inferred by comparison with  $\Phi$  or 1Kb  $\lambda$  molecular weight markers, were as well the expected ones in all the cases.

Where no band was obtained (*Halococcus morrhuae* NCMB 2012 and strain no. 704), regardless to the set of primers used, was probably due to the fact that in this cases the concentration of DNA was very low but the same dilution was applied (1:1000).

Table 1. PCR products obtained using the primer sets 008F-1517R and 007F-1517R.

Strain	008F-1517R	007F-1517R
<i>Haloferax volcanii</i> NCMB 2012	-	+
<i>Haloarcula marismortui</i> ATCC 43049	-	+
<i>Halococcus morrhuae</i> CCM 537	-	-
<i>Halomonas elongata</i>	+	-
<i>Haloarcula vallismortis</i> ATCC 29715	-	+
<i>Halomonas meridiana</i>	+	-
<i>Haloarcula hispanica</i> ATCC 33960	-	+
no. 704	-	-
<i>Micrococcus halobius</i> ATCC 21727	+	-
<i>Marinococcus halophilus</i> DSM 20408	+	-
no. 617	-	+
<i>Halobacterium salinarium</i> CCM 2148	+	+
Salt sludge enrichment	+	-
Blue salt enrichment	n.t.	-
White salt enrichment	n.t.	+
Control	-	-

+: one single band, -: no band, n.t.: not tested.

In the case of the DNA obtained from *Halobacterium salinarium* CCM 2148 an amplification band was obtained with the 007F-1517R primers but also with the other set suggesting a contamination of the original pure culture with a moderate halophile. This fact was confirmed by microscopic examination and isolation on plates.

Unluckily the rest of the project could not be finished due to a lack of time motivated by an unexpected malfunctioning of the PCR machine.

## CONCLUSIONS

The results so far obtained are good enough to think that this project should be continued in the near future aiming to get all the initial objectives. In any case it has been shown that the environmental samples used (even the marine ones) contain enough halophiles to be detected not only by isolation but also using rRNA molecular analysis.

## ACKNOWLEDGMENTS

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# **A study of the diversity of microbiota in the accessory nidamental gland of squid *Loligo pealei***

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## **Abstract**

One of the intriguing symbiotic topics in Microbiology is squid and their bacterial symbiont. Sepiolid squid *Euprymna scolopes* and its light organ bacterial symbiont *Vibrio fischeri* has been well described in the previous reports. However, the association of non-luminescent bacteria with the accessory nidamental gland of loliginid squid *Loligo pealei* has not been described in details before. In this project, I used microscopy as well as 16S rRNA-directed probes with fluorescently tagged oligonucleotide and extraction of DNA directly from the homogenized gland to study the diversity of microbiota that present in this squid's accessory nidamental gland. Using microelectrodes, I am able to detect the oxygen content and pH of the interior of the gland. I also tried to cultivate different bacteria from the gland and characterized these pure culture. Overall, this report showed that unlike *Euprymna scolopes'* light organ, the bacteria residing in the accessory nidamental gland of *Loligo pealei* have a quite diverse characteristics.

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**July 27, 1995**

**Microbial Diversity Course, 1995.**

**MBL, Woods Hole, MA.**

## Introduction

An intriguing topic of microbiology recently is the association of bacteria with "higher" plant and animal life. This includes "pathogen-host" relationship as well as symbiosis between bacteria and their host. One example of the latter is a small sepiolid squid, *Euprymna scolopes*, and its light organ bacterial symbiont *Vibrio fischeri*. This luminous bacterium colonizes the light organ and makes *E. scolopes*' bioluminescent. The light organ maintains a pure culture as many as  $10^8$  *V. fischeri*.<sup>1</sup>

Another squid *Loligo pealei* also has bacterial symbiont. Unlike *Euprymna*, however, *Loligo pealei* does not have a light organ and luminous bacterial symbionts. *L. pealei* is abundant in the sea around Cape Cod and Cape Hatteras, along the East Coast of United States. These animals are typically restricted to continental shelves and coastal margins and do not migrate through deep water.<sup>2</sup> Although *L. pealei* does not have a light organ containing bacterial symbionts, the accessory nidamental glands of female squid are heavily colonized by bacteria.<sup>3</sup> Unlike the bacterial population in light organ of *E. scolopes*, the bacteria present in the accessory nidamental gland are of quite diverse characteristics. But detailed examination of the microbiota of this gland has not been reported.

Using microelectrodes, I will first determine the oxygen content and pH of the interior of the accessory nidamental gland. A rapid method for the identification of bacterial cells using 16S rRNA-directed, fluorescently tagged oligonucleotide probes, will also be employed. Direct extraction of DNA from gland samples and characterization by comparative rRNA sequence analysis will also be used. Some isolations of pure cultures in different media such as sea water complete (SWC) medium, casamino acid-sea water (CAA-Sea) medium, yeast extract-glucose seawater (YEG-Sea) medium and VB2 are obtained during this study. With this information, we may begin to understand the diversity of the microbiota present in this squid's accessory nidamental glands.

## Materials and Methods

### Microscopy of accessory nidamental gland of *Loligo pealei*

*Loligo pealei* were obtained from Marine Resources Center, Marine Biological Laboratory, Woods Hole, Massachusetts. The accessory nidamental glands were removed from the squid. Each gland was homogenized and then observed under a phase contrast microscope. Examination of F<sub>420</sub> autofluorescence, chlorophyll autofluorescence and green autofluorescence were also employed.

### Enumeration and cultivation of bacteria of interior of the accessory nidamental gland

The total number of bacteria in accessory nidamental gland was estimated by direct microscopic count. Series of dilutions of homogenized gland were made and a proper dilution was counted using a Petroff-Hausser counting chamber.

Several different media were used to isolate bacteria from the gland. The media used included: Yeast extract glucose-sea water [YEG-sea, sea water, 70% (v/v); yeast extract, 1% (w/v) and glucose, 1% (w/v)], casamino acid-sea water medium [CAA-sea, sea water, 70% (v/v); casamino acid, 2% (w/v); K<sub>2</sub>HPO<sub>4</sub>, 0.1% (w/v) and yeast extract, 0.05% (w/v)], sea water complete medium [SWC, sea water, 70%(v/v); Bacto-peptone, 0.5% (w/v); yeast extract, 0.3% (w/v) and glycerol, 0.3% (v/v)]. Solid media were the same composition as above except 1.5% (w/v) agar was added in YEG-sea and CAA-sea media and 2% (w/v) agar was included in SWC medium. Another solid medium used was VB2 [NaCl, 1.3% (w/v); MgCl<sub>2</sub>, 0.2% (w/v); KCl, 0.07% (w/v); nutrient agar, 2.3% (w/v); yeast extract, 0.5% (w/v); Difco agar, 0.5% (w/v) and 0.1% (v/v) of ferrous ammonium sulfate solution (3% w/v)]. The accessory nidamental gland was sterilized by dipping in ethanol before homogenization to prevent other bacterial contamination from non-gland's source. Homogenized gland after dilution was either spread or streaked on the media and incubated aerobically and anaerobically in a Gas Pak jar at room temperature. After

growth, a single isolated colony was picked and restreaked on appropriate medium, then incubated at room temperature aerobically and anaerobically. Pure cultures were obtained after several transfer and then been characterized. The tests for characterization included: Gram stain, morphology, transmission electron microscopy, motility, oxidase, catalase bioluminescence, nitrate reduction and growth in different media and conditions.

#### Determination of oxygen content and pH of the interior of the accessory nidamental gland by microelectrodes

Oxygen content of the interior of the accessory nidamental gland was determined by a Clark microelectrodes with a guard cathode. pH inside the gland was measured by a pH microelectrode constructed from pH-sensitive glass and lead glass.<sup>4</sup> The oxygen was measured using intact gland while measuring pH, the gland was cut open and the interior of the gland was measured due to the fragility of the pH microelectrode and its inability to penetrate the intact gland. As a control, other parts of the squid such as gills and nidamental gland were also tested for oxygen content.

#### Direct extraction DNA, amplified by polymerase chain reaction, cloning and sequencing rDNA and identity

Bacterial DNA was extracted directly from homogenized accessory nidamental gland using "DNA extraction from environmental samples" method (J. Dörr, unpublished data) followed by "hot start" polymerase chain reaction procedure to amplify rDNA of the bacteria in the environmental sample (the gland). Purified PCR product (rDNA) was then subjected for ligation into a commercial vector pCR II<sup>R</sup> and transformed into competent cells (*E. coli*) using a commercial kit, Original TA cloning<sup>R</sup> kit (Invitrogen Co.) Due to time restriction, Sequencing and identity were not employed.

### Testing for autoinducer produced by bacteria in accessory nidamental gland

Homogenized accessory nidamental gland (the pure cultures from the gland) was streaked in the center of SWC agar. *Vibrio fischeri* MJ211 ( $\Delta luxI$  strain) was obtained from Dr. Paul Dunlap (WHOI, Woods Hole, MA).<sup>5</sup> This mutant can still luminesce if stimulated by exogenous autoinducer but can not produce its own autoinducer. MJ211 was streaked alongside a streaked line of homogenized gland (or pure culture isolated from the gland), either at the same time or after the growth of bacteria from gland occurred. (Fig A). The plates then were incubated at room temperature (23°C- 25°C). The appearance of luminescence was observed after *Vibrio fischeri* MJ211 grew. If the bacteria from the accessory nidamental gland produced autoinducer, MJ211 should exhibit luminescence.

### *In situ* hybridization using 16S rRNA-directed, fluorescently tagged oligonucleotide probes

Pure cultures isolated from accessory nidamental gland were grown in SWC liquid media overnight and 1 ml of culture was centrifuged and washed with PBS (pH 7.4 ) twice and resuspended in 1 ml PBS. Cells were then diluted 1 : 100 with PBS and 15  $\mu$ l were spotted onto subbed, baked, 10-well slides (Cell-Line Associates, Newfield, NJ). Environmental samples (homogenized gland) were also employed by the similar method. The cells were then fixed with ethanol/formaldehyde (90/10, v/v) and hybridized with 16S rRNA-directed, fluorescently tagged oligonucleotide (Rhodamine labeled) probes overnight in appropriate temperature (37°C or 45°C). The probes used here included universal probe and probes detecting the following group of bacteria: High G+C bacteria; Low G+C bacteria; Proteobacteria: Alphas group; Betas group, Deltas group and Enteric (Gammas Group); Pseudomonades and Flavobacteria. The results were examined under a microscope with fluorescent filter. The detail procedure was described in reference 6.

## Results and Comments

Accessory nidamental glands are paired organs founded in female *Loligo*, have been assumed to play a secretory role associated with the reproductive system (Fig B), and their color can be used as a marker for sexual maturity of the squid.<sup>2</sup> *Loligo pealei* house symbiotic bacteria within their accessory nidamental gland has been reported but not in details.<sup>3</sup> The results here show that some characteristics of the bacteria residing in the gland.

### Microscopy of accessory nidamental gland

The results of microscopy of the homogenized gland are shown in Fig C-1 (differential interference contrast microscope) and Fig C-2 (phase contrast microscope). The results shown a mixed population of bacteria residing in the gland with different shape (rod, short rod, cocci, etc.) and motility ( both motile and nonmotile). All the results of autofluorescent examination were negative (no autofluorescence beed observed).

### Emumeration and cultivation of bacteria of the interior of accessory nidamental gland

The total number of bacteria in the accessory nidamental gland is about  $2.3 \times 10^{11}$  bacteria/ml (the data is from the average of three different counts of one squid). The results of isolation of bacteria and some of their characteristics are summarized in Table 1. There were six isolates studied in this report: strains SQ1, SQ2, SQ3, SQ4, SQ5 and SQ6. The microscopy results are shown in Fig C-3 to Fig C-8. Results of transmission electron microscope of strains SQ, SQ2, SQ3, SQ4 and SQ5 are shown in Fig D-1 to Fig D-5.

### Oxygen content and pH of the interior of the accessory nidamental gland

Oxygen content shown immediately drop to totally anoxic inside the gland of a fresh sacrificed squid. The control parts of the squid (e.g. gills and nidamental gland) also shown anoxic. However, squids are highly metabolic animals and after sacrificed their circulation and exchanging of oxygen stopped. Also the oxygen in the accessory nidamental gland could be depleted by those bacteria residing in the gland since all the isolates in this study could grow aerobically. pH of the interior of the gland was about neutral (pH about 6.8) determined by pH microelectrode.

#### Direct extraction DNA, PCR, cloning, sequencing rDNA and identity

Bacterial DNA extracted directly from accessory nidamental gland was successfully obtained. The DNA was amplified by "hot start" PCR procedure and purified and then inserted into a vector pCR II. Unfortunately, I did not get any transformed cells in the transformation step. I had repeated three times, two of them I did not get any cell grew and the last time I got a confluent growth. The confluent growth might due to the improper preparation of plates with kanamycin and so kanamycin did not select the transformed cells. From the confluent growth plates, I washed the cells off and then spread on another kanamycin-LB plate. I got some presumably transformed colony from this plate. In order to confirm if these are transformed cells I will have to do the plasmid preparation. However, since time is limited here I was not allow to do any further experiments for this rDNA extraction and comparative.

#### Autoinducer production by bacteria in accessory nidamental gland

The results shown none of the isolates as well as homogenized gland produce autoinducer complement to that of MJ211. However, this experiment only used *V. fischeri* as reference bacteria. Since autoinducer is species-specific, I do not know if they produce autoinducer that complement to other luminescent bacteria such as *Vibrio harveyi* or *Photobacterium sp.*

### *In situ* hybridization with 16S r-RNA directed, fluorescently tagged oligonucleotide probes

All the samples reacted with universal probes but the environmental sample (homogenized gland) reacted much weaker compared with those of pure culture. Strains SQ1, SQ2 and SQ6 reacted with Enteric ( Gamma) group probes. Since environmental sample reacted much weaker with universal probe, the reason that no Enteric probe reacted with environmental sample may due to the reaction was too weak to be detected. All the rest of the probes shown no reaction with any of the samples. (Table 1) However, the results of the control for the probes for low G+C bacteria, Alphas and Deltas group did not shown positive, either.

Summary: The results above shown a quite diverse characteristics of the microbiota residing in the accessory nidamental gland of *Loligo pealei*. From 16S rRNA probes and the characteristics of the isolates, I could conclude at least some of the bacteria are belong to the Family Vibrionaceae (e.g. SQ1, SQ2 and SQ6).

In this study, I did not try to isolate any of the strict anaerobic bacteria from the gland. Since the interior of the gland of a fresh sacrificed squid shown totally anoxic, it would be worthwhile try to isolate some anaerobic bacteria as well as facultative ones from the gland in the future.

The direct extraction of DNA from environment and comparative rDNA sequences will help us to understand the diversity of the microbiota in the gland and can compared to those of isolates' rDNA sequences to see if there are any agreements. Since the restriction of the time here, I did not have chance to complete these experiments.



Table 1: Some characteristics of the isolates from squid accessory nidamental gland

isolate	SQ1	SQ2	SQ3	SQ4	SQ5	SQ6
initial isolate media and condition	aerobic, CAA-sea	aerobic, SWC	aerobic, SWC	aerobic, YEG-sea	aerobic, YEG-sea	anaerobic, CAA-sea
morphology	rod(curve) cocci	rod-cocci	rod	rod-cocci	big rod	big rod
flagellated (motility)	yes	yes	no	no	no	yes
Gram stain	negative	negative	negative	negative	negative	negative
oxidase	positive	positive	positive	positive	positive	positive
catalase	positive	positive	positive	positive	positive	positive
media that can grown with aerobically	CAA-sea, YEG-sea, VB2, SWC	CAA-sea, YEG-sea, VB2, SWC	YEG-sea, VB2, SWC	YEG-sea, VB2, SWC	YEG-sea, VB2, SWC	CAA-sea, YEG-sea, VB2, SWC
growth at 37 C	no	no	no	no	no	no
reacted with 16S rRNA probes	universal, Enteric probes	universal, Enteric probes	universal probe only	universal probe only	universal probe only	universal, Enteric probes
growth** in YEG-sea anaerobically	+/-	-	-	-	-	+/-
growth** in SWC anaerobically	+	+/-	-	+/-	-	+
growth **in SWC + 1% nitrate anaerobically	+	+/-	+/-	+/-	+/-	+

\* All of the isolates do not grow well (or not grow at all ) on YEG and CAA without sea water base.

\*\* When growth occurred, the pH of the media dropped to about pH 5.

\*\*\* All strains shown only background growth on VB2 plates anaerobically.

Figure A: Streaking for testing autoinducer

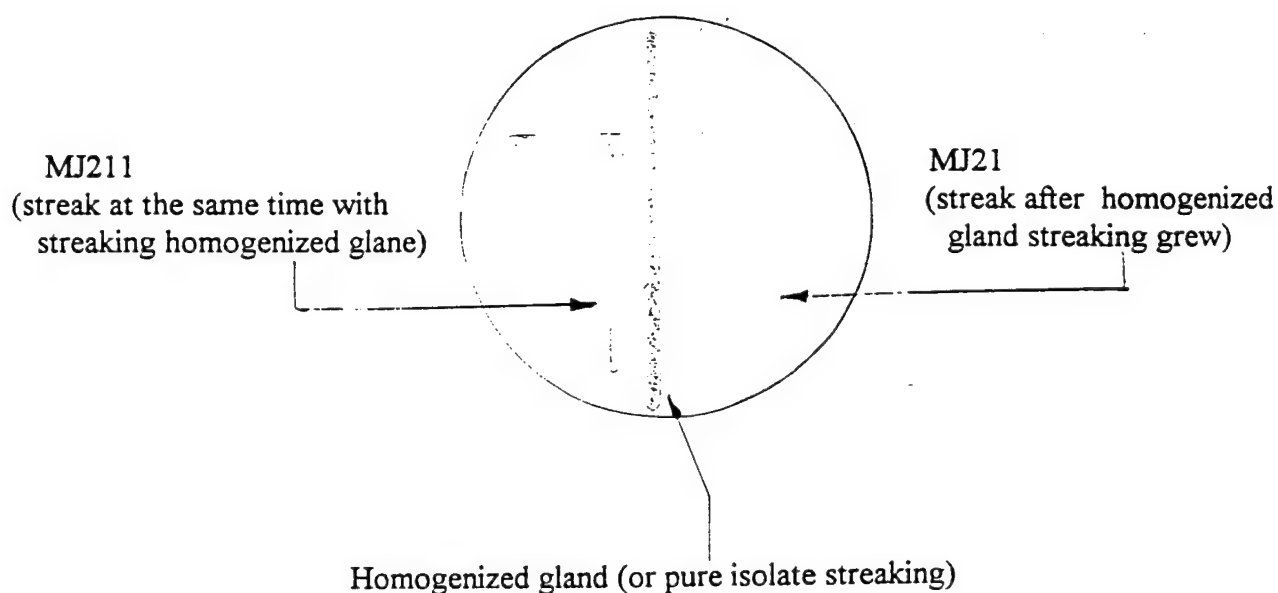


Figure B: The squid *Loligo pealei* and accessory nidamental gland

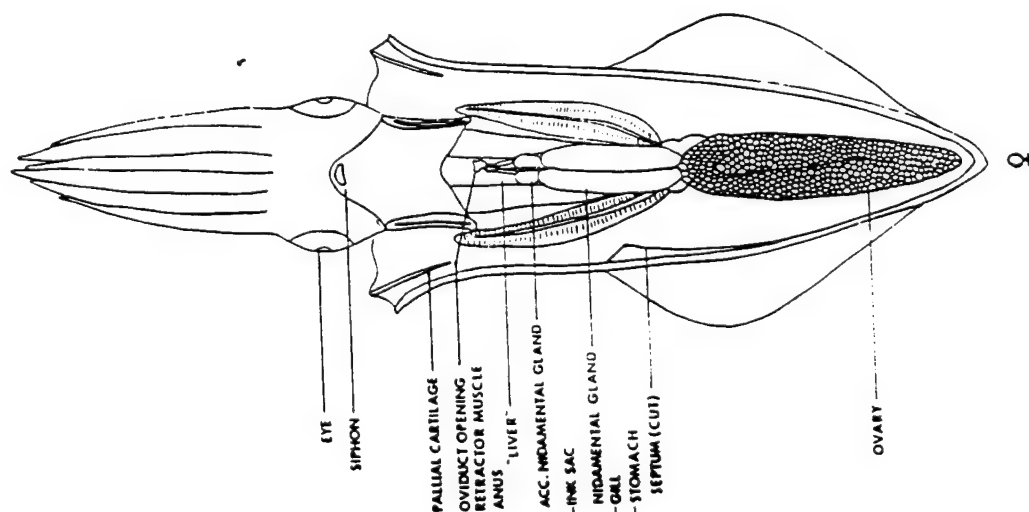


Figure legends:

Fig C:

Fig C-1: homogenized accessory nidamental gland under DIC microscope

Fig C-2: homogenized accessory nidamental gland under phase contrast microscope

Fig C-3 to Fig C-8: Strains SQ1, SQ2, SQ3, SQ4, SQ5 and SQ6 under phase contrast microscope, respectively. Strain SQ1, SQ2 and SQ6 shown highly motility while others did not.

Fig D: photograph of bacteria isolated from accessory nidamental gland under transmission electron microscope using negative staining

Fig D-1 and Fig D-2: Strain SQ1, Shown vibrio shap with flagella

Fig D-3: Strain SQ2, rod shape

Fig D-4: Strain SQ3, without flagella

Fig D-5: Strain SQ4, short rod without flagella

Fig D-6: Strain SQ5, the long rod in the center of the picture might be a contamination of the preparation.

Figure C: Figure C-1 to Figure C-4

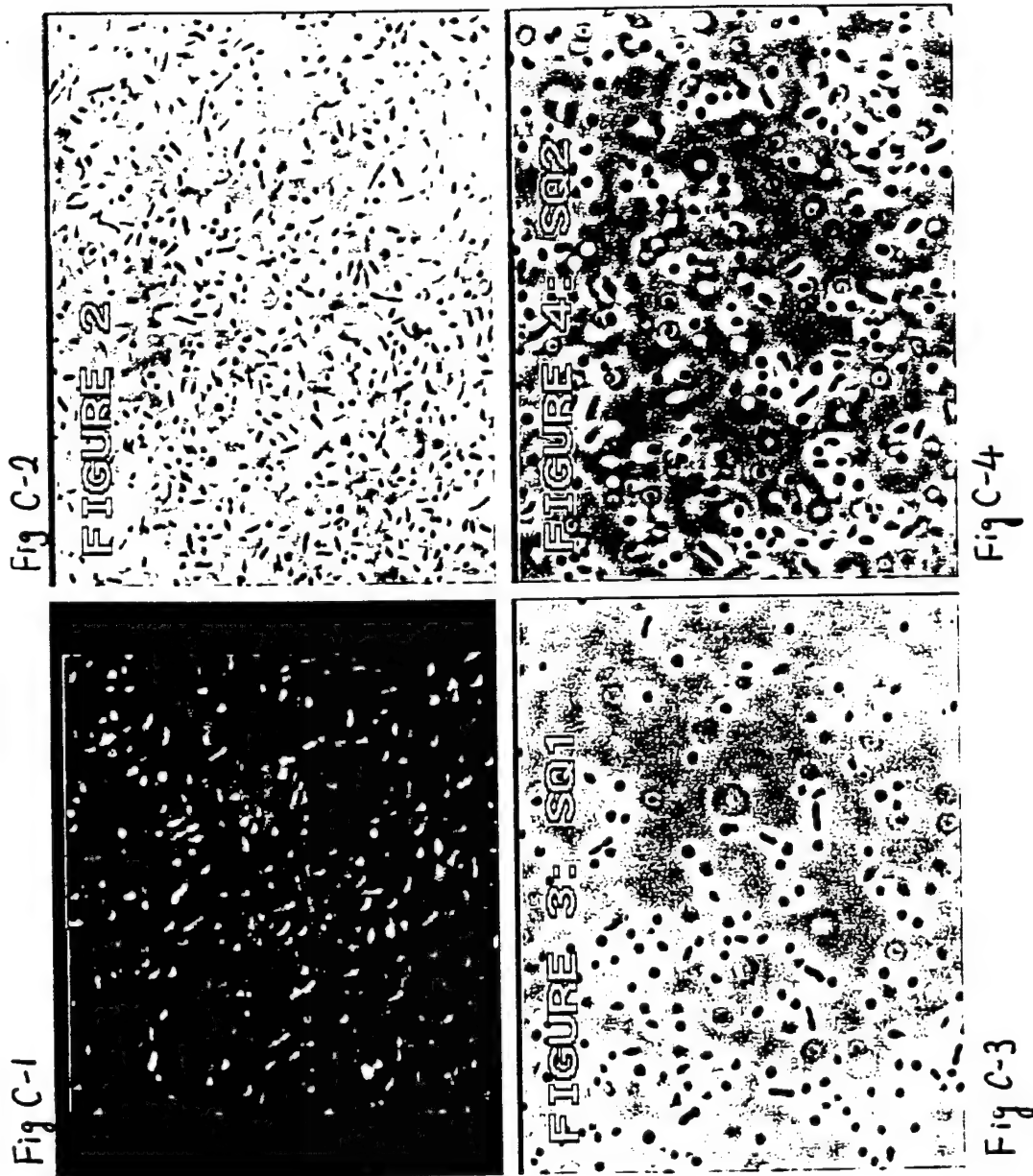


Figure C: Figure C-5 to Figure C-8

Fig C-5



Fig C-6

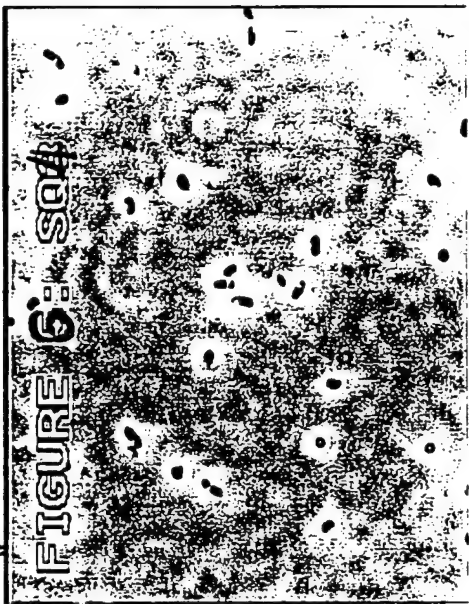


Fig C-7



Fig C-8

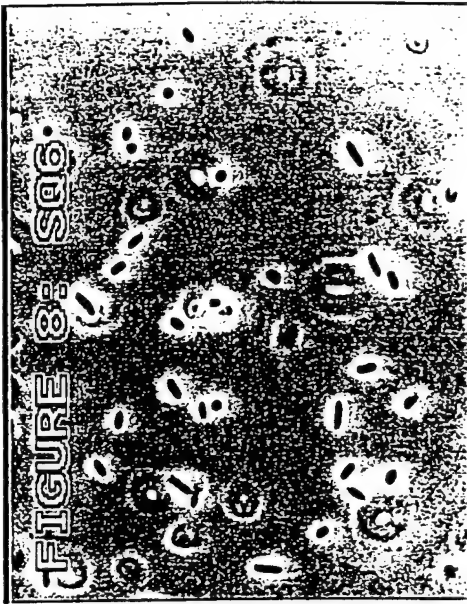


Figure D: Figure D-1 to Figure D-3

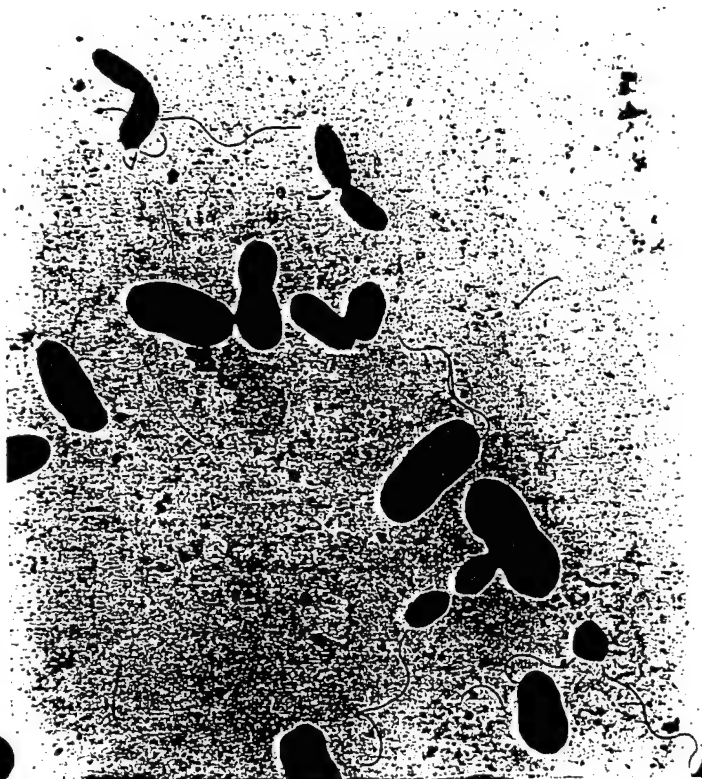


Fig D-1

Fig D-1

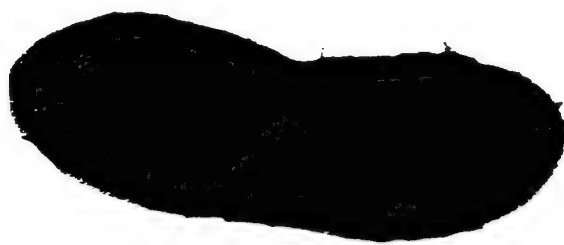


Fig D-2

Fig D-2

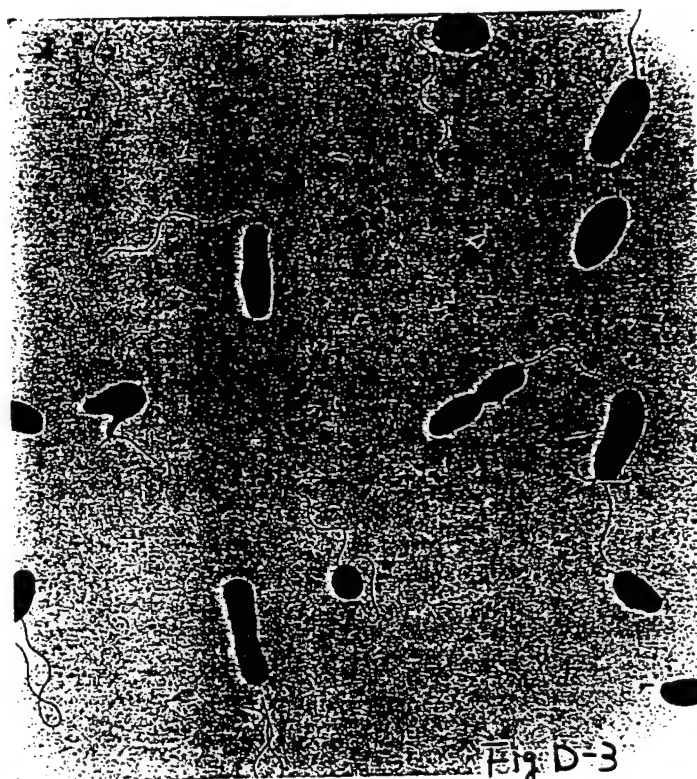


Fig D-3

Fig D-3

Figure D: Figure D-4 to Figure D-6



Fig D-4



~~Fig D-5~~  
Fig D-5

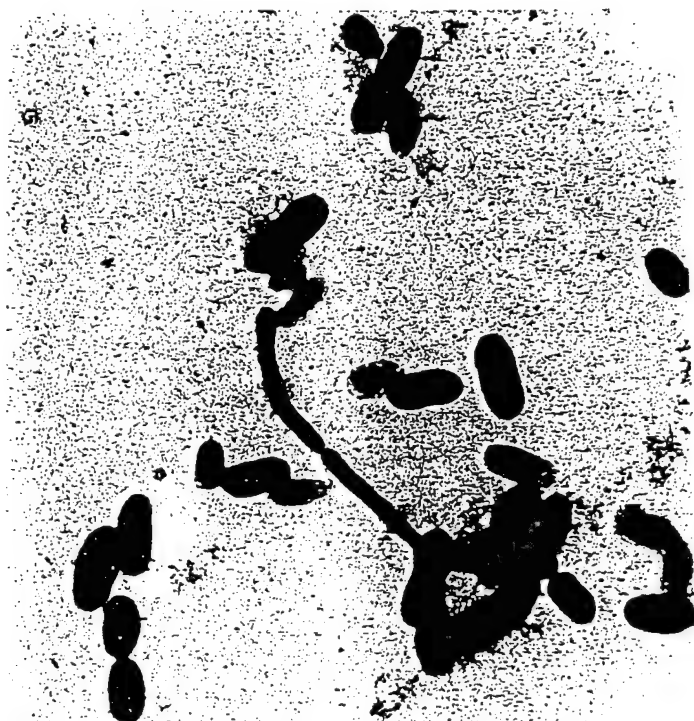


Fig D-6

Fig D-6

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6. Braun-Hawland, E.B., S.A. Danielsen and S.A. Nierzwicki-Bauer. 1992. Development of a rapid method for detecting bacterial cells *in situ* using 16S rRNA-targeted probes. *BioTechniques*, 13(6): p. 928-933.



# Microbiota in the Intestinal tract of marine fish *Fundulus heteroclitus*

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Microbial Diversity Course, 1995, MBL, Woods Hole, MA.

University of Connecticut, Storrs, CT.

*Fundulus heteroclitus* is a stout-bodied little fish (Fig 1), they inhabit along sheltered shores where the tide flow over beds of eelgrass or salt hay. They can fairly be characterized as "universal" in suitable location around the entire coastline of the Gulf of Maine and are favorite for biological experiment.(1)

Gut microbiota has been an intriguing topic in environmental microbiology, but most of the studies were focus on mammalian intestinal tract and aquatic invertebrates. (2) Nearly none of the report has been described for marine fish gut microbiota. In this project, I tried to examine the gut microbiota of *Fundulus heteroclitus*.

Using phase contrast microscope and DIC microscope, I examined the gut content as well as gut wall itself to see if any significant bacterial population colonized inside the gut. Suprisingly I could not find any significant microbial population from the gut samples. (Fig 2) I also did 16S rRNA-directed, fluorescently tagged oligonucleotide probes hybridization (3), but no positive reaction was observed including universal probes. In some attempt by enrichment technique to isolate bacteria using different media (e.g. Sulfate reducing bacteria medium, yeast extract-glucose medium, TYG [a medium for Bacteroid]), I got different results from every different time and it seemed those were come from contamination. The initial conclusion for this project now is that there is no heavily colonization of bacteria in *Fundulus heteroclitus'* gut. It is not unusual since some marine isopod species have also been reported that absence of microorganisms in their digestive tracts.(4)

Since the absence of microbiota in the gut, I tested if the gut content had inhibition effect to bacterial growth. I spread a mix bacteria (isolated from sea water where the fishes lived) on the SWC (sea water complete) agar plates as the lawn and lay a cut open gut on the plate. There was no inhibition zone after incubation. No further experiments has done after this. A suggestion for the experiment in the future will be using different concentration of bacterial lawn and using pour agar plate method instead of spread agar method.

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Figure 1: *Fundulus heteroclitus*

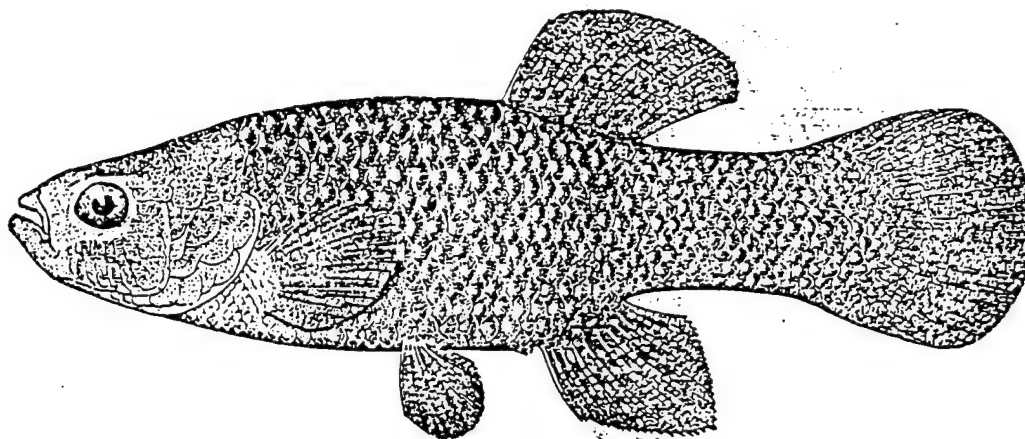
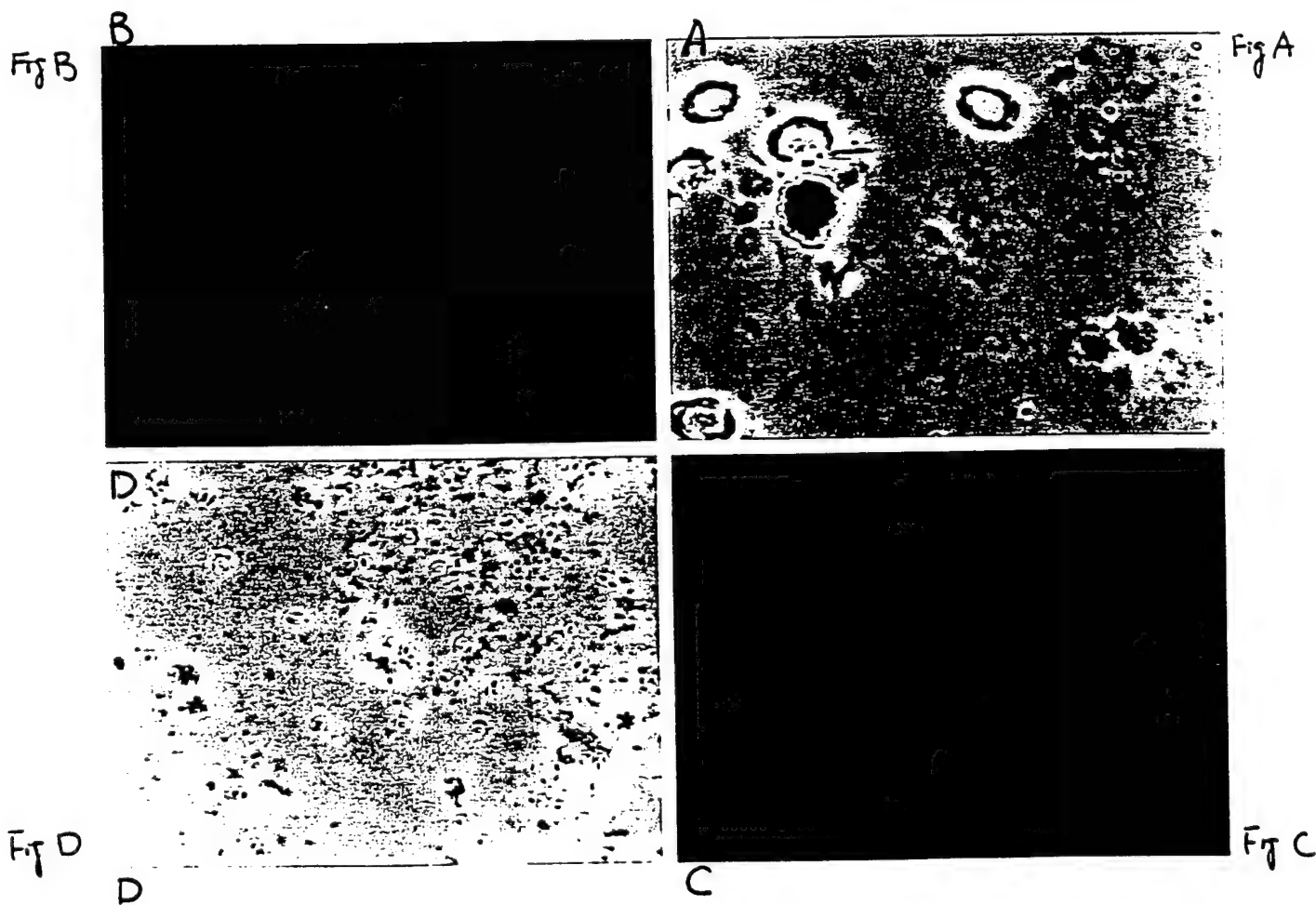


Figure 2: Examination of gut content under phase contrast microscope (A&D) and by 16S rRN directed probes. Both shown no major bacterial population present.



# Microbial transformation of sulfide and 3-mercaptopropionate.

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**Abstract.** Gradient tubes and deep agar shakes were made to enrich for chemotrophs and phototrophs able to grow on sulfide or 3-mercaptopropionate (MPA) as electron donor and with  $O_2$ ,  $NO_3^-$  or photosynthesis as electron acceptor. Enrichment of bacteria seemed to be possible for all the metabolisms yielding different rods, cocci and spirilla for the chemotrophic growth, purple non-sulfur bacteria for phototrophic growth on MPA - of which one isolate exhibited scotophobic response - and purple sulfur bacteria for phototrophic growth on sulfide.

## Introduction.

The redox cycle of sulfur in nature includes biological as well as chemical transformations, making the study of the fate of sulfur quite complex. The sulfur redox states commonly found on Earth are: -2 (sulfhydryl and sulfide), 0 (elemental sulfur) and +6 (sulfate). Inorganic sulfur compounds can be reduced (sulfate) or oxidized (sulfide and elemental sulfur) by sulfate reducing bacteria and sulfur oxidizing bacteria, respectively. Organic sulfur compounds synthesized by living organisms enter into the biogeochemical sulfur cycling for its degradation.

Most organosulfur compounds in marine environments originate from the degradation of dimethylsulfoniopropionate (DMSP). This compound is present in many plants and algae, especially marine living forms, where it is believed to function as an osmoregulator. DMSP occurs in sediments of shallow coastal environments due to the accumulation of algae and plant detritus. DMSP may degrade to 3-mercaptopropionate (MPA) in one of two ways: by two successive microbially mediated demethylations or by the chemical addition of  $H_2S$  to acrylate, which is an alternative microbial product from breakdown of DMSP. The  $H_2S$  used in the latter pathway may also originate from the breakdown of DMSP, but  $H_2S$  is also an abundant end-product from sulfate reduction.

$H_2S$  has long been known to be transformed by a variety of organisms. Colorless sulfur bacteria chemolithoautotrophically or organolithotrophically oxidize  $H_2S$  with oxygen or nitrate and purple sulfur bacteria utilize  $H_2S$  as the electron donor in anoxygenic photosynthesis.

The only report of a pure culture capable of utilizing MPA came from Visscher and Taylor (1988), who found that *Thiocapsa roseopersicina* could cleave the sulfide group of MPA and using it as electron donor in anoxygenic photosynthesis. In this organism the carbon backbone of MPA was not utilized, that is the organisms grew photolithoautotrophically. Thermodynamically, MPA could probably be converted by oxidation with both  $O_2$ ,  $NO_3^-$ , and  $SO_4^{--}$  as well as by photoheterolithotroph organisms utilizing the mercapto group as electron donor and the carbon backbone as a carbon source. None of these have been isolated so far.

Knowledge of the organisms responsible for the degradation of MPA obtained by isolation is important in the understanding of the regulation of the sulfur cycle. The isolation of a denitrifying organism capable of utilizing MPA would provide a potential new link between the sulfur and nitrogen cycles in marine habitats.

In this study we will try to isolate some not previously described microorganisms responsible for the underlying conversions.

## Materials and Methods.

### *Inocula.*

Five different inocula were used. 1) Pin: pink material collected at Sippewissett Salt Marsh on June 13 consisting almost entirely of 20-30  $\mu\text{m}$  long flagellated rods organism containing sulfur granules in the cytoplasm (Fig. 1). After collection the sample was stored at 4°C until inoculation 3 weeks later. Microscopy revealed no changes in the morphology of the cells as a consequence of storage. 2) Car: Sediment collected June 24 from Oyster Pond containing a high number of large sperical flagellated cells with cytoplasmatic granules, which was suspected to give the inoculum its purple colour. 3) Con: Sample from Sippewisset Salt Marsh collected on June 13 containing orange-coloured material. The sample was stored in a glass jar in light at room temperature for 3 weeks before inoculum was scraped from the sides of the glass. Microscopic examination prior to inoculation revealed sheets of small cocci. 4) SES: Surface mud collected at 20 m depth in Cape Cod Bay on July 2. Until inoculation the sample was stored at 4°C in the dark. 5) Wrm: Worm tube collected at 20 m depth in Cape Cod Bay on July 2.

### *Media.*

For initial enrichments in gradient tubes and for deep agar shakes was used a mineral salts medium for purple sulfur bacteria:

per liter:	
$\text{KH}_2\text{PO}_4$	0.25 g
$\text{NH}_4\text{Cl}$	0.34 g
KCl	0.34 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.50 g
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	2.80 g
NaCl	20.0 g
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.25 g

For bacch cultures a slightly different mineral salts medium was used:

per liter:	
$\text{KH}_2\text{PO}_4$	0.20 g
$\text{NH}_4\text{Cl}$	0.25 g
NaCl	20.0 g
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	3.00 g
KCl	0.50 g
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.15 g

The salts solutions were autoclaved in Widdel flasks and cooled under a 80/20  $\text{N}_2/\text{CO}_2$  atmosphere. Then the following sterile solutions were added:

per liter:	
$\text{NaHCO}_3$	18 mM final
6-vitamin solution	1.0 ml
Vitamin $\text{B}_{12}$ -solution	1.0 ml
Trace elements SL12	1.0 ml

and pH was adjusted sterilely to 7.2.

Of this solution 175 ml was tapped anaerobically into a bottle and tightly sealed before 1.07 ml of 1 M sterile  $\text{Na}_2\text{S}$  or MPA was added to make 1.25 mM final concentration. pH was adjusted again to 7.2 with sterile HCl by measuring pH in small aliquots after additions. Eight Phennig bottles were filled anaerobically with a total of 440 ml of this solution before another 1.57 ml of 1 M  $\text{Na}_2\text{S}$  or MPA were added to yield a final

concentration of approximately 5 mM. Of this solution, 175 ml were filled anaerobically into a bottle, which was then closed airtight.

Agar, washed 4 times in deionized water, was made up in concentrations of 1.25% and 7.5 % and autoclaved.

#### *Gradient tubes.*

While still hot after autoclaving, aliquots of 1 ml of 12.5% agar was tapped into 20 ml test tubes, which were placed in a 60°C water bath. Four ml of medium containing 5mM of sulfide or MPA was added after being preheated for 10 min. in a 40°C water bath. The test tubes were then flushed with 80/20 N<sub>2</sub>/CO<sub>2</sub>, closed airtight and cooled to roomtemperature in a water bath so that a solid plug of 1.5% agar containing 4mM sulfide or MPA was formed. The 1.25% agar was placed in a 60° water bath while still hot after autoclaving. The medium without sulfide or MPA was warmed up to 40°C in a water bath and mixed anaerobically with the 1.25% agar to yield a top agar with a concentration of 0.25%. Eight ml of this agar was then poured anaerobically on top of the plug in each of the test tubes to form a slush agar overlay. The test tubes were then left for three days to allow opposing gradients of oxygen and sulfide or MPA to establish through the top agar before inoculations. Nitrate gradient tubes were made by adding an anoxic 3 ml water phase containing 2 mM NaNO<sub>3</sub> after inoculation and subsequently flushing the headspace with 80/20 N<sub>2</sub>/CO<sub>2</sub> before closing the tubes airtight. That means that the top agar in the nitrate gradient tubes initially contained som oxygen as they were left for 3 days under atmospheric air.

Inoculations were made by filling approximately 0.2 ml of inoculum into a Pasteur pipette which had previously been pulled in a flame. The pipette was then inserted into the agar down to the top of the agar plug and retracted while slowly dispensing the inoculum in the entire length of the top agar.

Microscopic inspection of growth was performed by pulling a Pasteur pipette in a flame, sucking out material with it from discrete locations in the tube and examining it under the microscope.

#### *Batch enrichments.*

Twenty ml of medium containing sulfide or MPA were anaerobically filled into serum bottles. The bottles had 1) the headspace flushed with atmospheric air or 2) 2 mM NaNO<sub>3</sub> the headspace flushed with 80/20 N<sub>2</sub>/CO<sub>2</sub> after inoculation and were sealed airtight before they were put in the dark. The inocula were the above described and material picked from the gradient tubes.

#### *Phototroph enrichment.*

Enrichment for phototrophic bacteria that can use sulfide or MPA was performed by making deep agar shakes in 6 fold 10 times dilution series. The enrichments were left in the dark for 24 hours to eliminate any potentially harmful oxygen, before they were placed in incandescent light behind a light filter that eliminated all light with wavelengths below 840 nm. The filter was applied to give purple sulfur bacteria a selective advantage. For purification Pasteur pipettes were pulled over a flame, colonies were picked and suspended in purple sulfur bacteria medium and inoculated in new deep agar shakes..

### Results.

#### *Gradient tubes.*

PIN:

*Sulfide:* All inocula responded with growth. Grown with oxygen PIN macroscopically at all depths showed pink clouds of cells, microscopically the cells were mostly small cocci with some short rods. Grown with NO<sub>3</sub><sup>-</sup>, less growth appeared and the colour was less pink. Again, the cells were small cocci mixed with some rods and spirals.

*MPA*: Growth was weaker in *MPA*-enrichments than with sulfide, and growth was macroscopically grey with only a weak pink tone to it. Microscopically mostly cocci were observed among some rods and spirals. Grown with oxygen characteristic fat rods appeared,  $5 \times 10 \mu\text{m}$  without granules in the cytoplasm.

**CAR:**

Tubes of all treatments got growth of an agar degrading organism, which dissolved all the slush agar within 10 days leaving only a clear liquid phase with some refractile material. Microscopically the cells were long (20-30  $\mu\text{m}$ ) slender rods.

**CON:**

Very weak growth on both sulfide and *MPA*. The few cells consisted of cocci and rods. There were some signs of slush agar degradation, which corresponded well with the observation of cells similar to the ones found in *CAR*. A special cell morphology was found with enrichment with sulfide and both  $\text{O}_2$  or  $\text{NO}_3^-$ : slender rods with a round "head" on one end. The enlargement was not refractile as typical endospores.

**SES:**

*Sulfide*: Only weak growth. With  $\text{NO}_3^-$  some very long (approximately 50  $\mu\text{m}$ ) slender rods, presumably gliding bacteria. Growth with oxygen produced short rods.

*MPA*: Almost no growth with *MPA*.

**WRM:**

Only tube with sulfide and oxygen showed a weak growth, consisting of cocci and rods. Other treatments showed close to no growth.

*Batch enrichments.*

No growth was visible after 10 days of growth.

*Phototroph enrichments.*

Good growth of colonies was observed in *PIN* and *CAR*, especially when grown on  $\text{H}_2\text{S}$ . *CON*, *SES*, and *WRM* had only few colonies. Of three morphologically different colonies isolated on  $\text{H}_2\text{S}$  all were large ovoid cells (Fig. 2) containing sulfur granules. Three colonies isolated on *MPA* from *PIN* and *CAR* were cocci, rods and spirilla, respectively, without visible intracellular sulfur granules. The spirillum was phototactic (Fig. 3).

Discussion.

This study suggests that some microorganisms are indeed able to degrade *MPA* in ways that has not been shown before. The gradient tubes showed heterotroph growth on both oxygen and  $\text{NO}_3^-$ , but did not create the expected result, that growth would develop as a band where opposing gradients would theoretically cross as has been shown by Nelson et al. In the present study the growth was not distributed in that manner, instead growth was diffuse in the entire length of the slush agar. This could be a result of the fact that the establishment of a gradient system is dependent on the activity of the bacteria and that the bacteria also are dependent of the establishment of the opposing gradients. If the bacteria are not growing fast enough to consume both electron acceptor (oxygen or  $\text{NO}_3^-$ ) and donor (sulfide or *MPA*), the acceptor can diffuse all the way down through the slush agar and the donor can diffuse all the way up, thus not creating the narrow overlap intended. Nelson et al. showed that chemical oxidation of sulfide would create almost as narrow an overlap, which would be only attenuated by the biological activity. That this was the case in this study as well was supported by the observation of bands of sulfur granules in the agar. In the case of *MPA* such a rapid chemical oxidation is probably not possible. In any case, microbial growth was not observed to form narrow bands.

A problem with the kind of gradient tubes used in this study is that making a thin slush agar to allow bacterial movement also makes the agar susceptible to degradation.



That the batch experiment did not produce any growth was unexpected. In the case of batch with both sulfide and  $O_2$ , chemical oxidation on the expense of microbial oxidation, but the combinations with  $NO_3^-$  and sulfide or MPA should not have this problem. Still no growth was observed, although growth on the same compounds seemed possible in the gradient tubes. A possible explanation could be that the gradient tubes which were supposed to contain  $NO_3^-$  and no  $O_2$ , were loaded with enough  $O_2$  during the preparation of the tubes (see materials and methods section) to sustain the amount of growth seen in the  $NO_3^-$  tubes. In that case the suggestions in this study, that both MPA and sulfide were oxidized with  $NO_3^-$  are maybe not correct.

Phototrophic growth was shown on both  $H_2S$  and MPA. In contrast to the finding that the purple sulfur bacteria *Thiocapsa roseopersicina* could utilize MPA, only purple non-sulfur bacteria were found to utilize MPA.

In all cases  $H_2S$  caused more growth than MPA. The precise faith of MPA in each case is unclear. The mercapto group may be cleaved of MPA and only the resulting sulfide or only the resulting propionate may be utilized by the organism.

The cells identified in the inocula PIN and CAR could not be retrieved in isolate by any of the enrichments used. The growth in gradient tubes with oxygen and  $H_2S$  showed pink growth which corresponded well with the large vacuolated cells of the inoculum, but the bacteria responsible for the colour were small rods which were not accumulating granules. This means that either a different pink organism than the most abundant in the inoculum grew up, or that the cells change their morphology as a response to growing in the dark. This could have been tested by transferring the cells to a purple sulfur bacteria enrichment system, but time didn't allow for this.

Further purification and control experiments are necessary to confirm the findings in this study.

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#### Legend:

Fig. 1. Electronmicrograph of presumably purple sulfur bacteria. Note the two polar tufts of flagella. The cell lysed during preparation, which shows as a dark outgrowth on the side of the cell. Another artefact is the empty space between cell body and the tufts of flagella, which is probably due to shrinking of the cell body or the flagella during preparation. Cell body approx. 25  $\mu m$  long.

Fig. 2. 40  $\times$  micrograph of a strain of purple sulfur bacteria.



Fig. 3. Scotophobic behavior in a strain of purple non-sulfur bacteria. The upper and lower panels represent two different levels of magnification. The panels to the left show the cells in a situation where the light diaphragm of the microscope is closed to only allow a small portion of the field of vision to be illuminated. The panels on the right show the situation approximately 1 sec. after the diaphragm was opened to the normal position. It is clear to see that the cells had accumulated in the lighted area. A few seconds later the cells were completely dispersed (Data not shown).

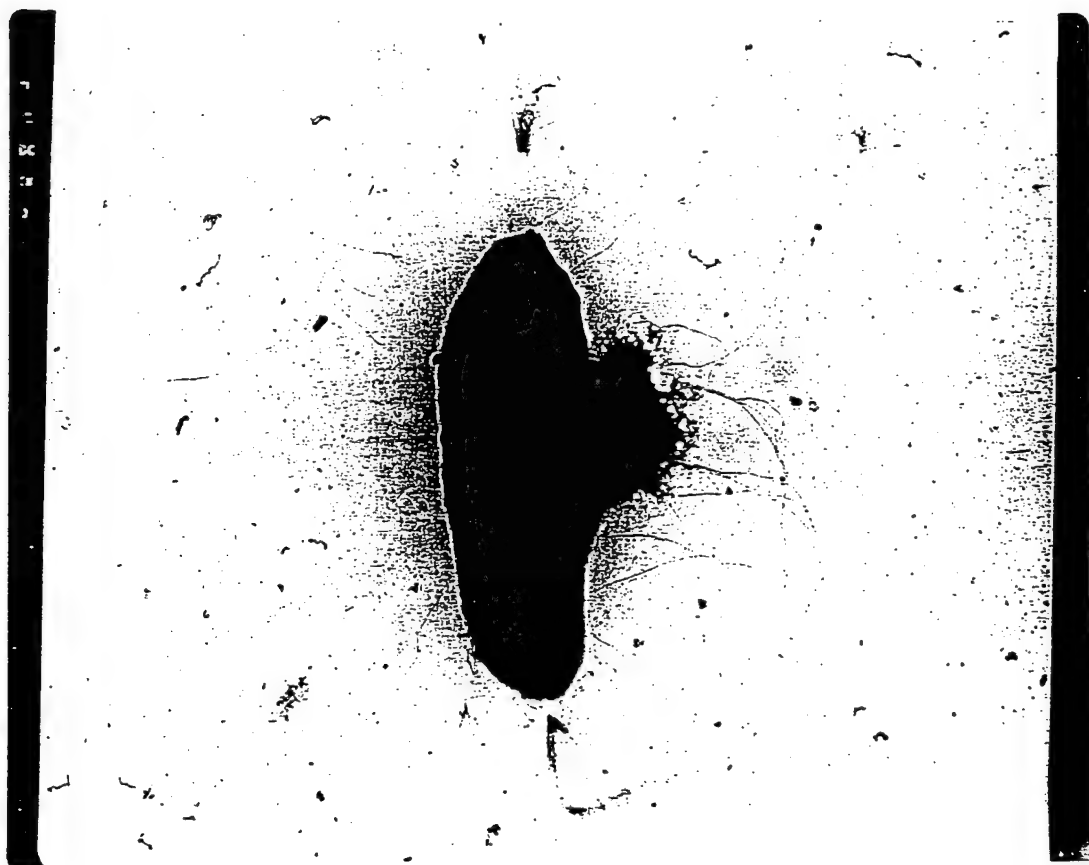


Fig 1.

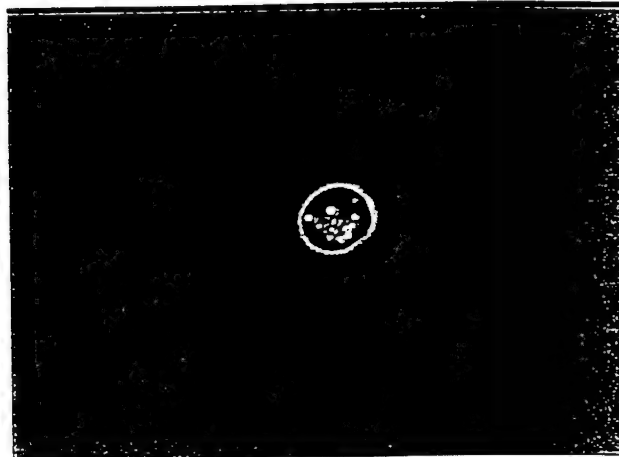
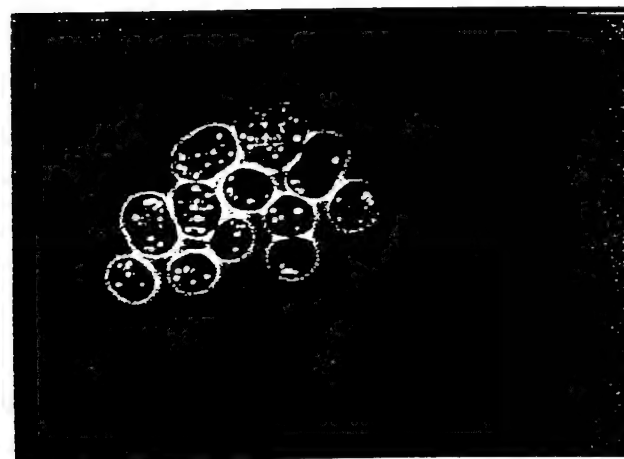
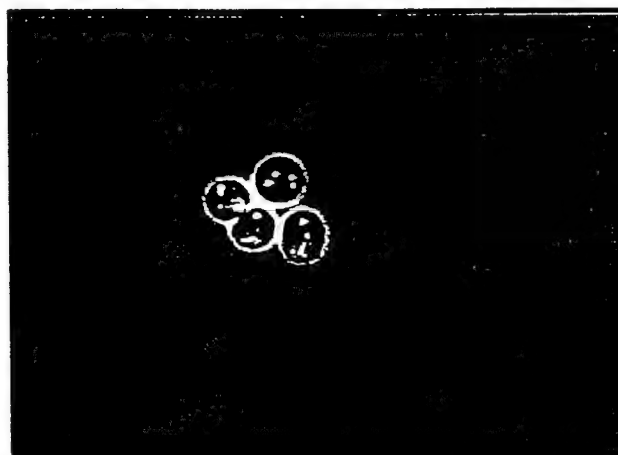


Fig. 2

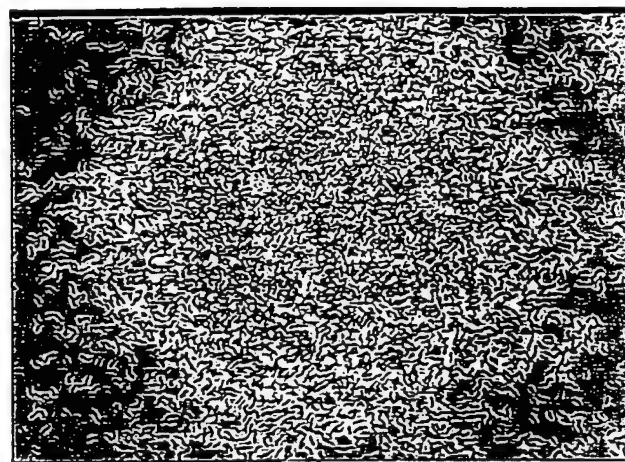
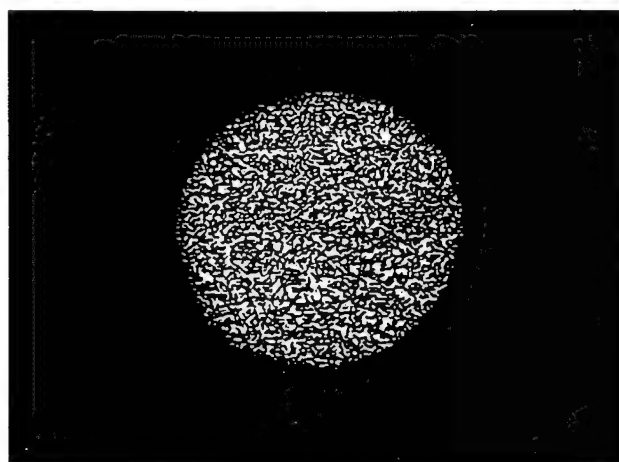
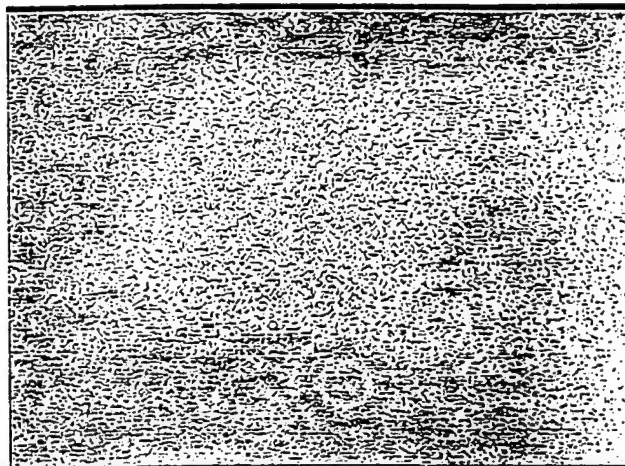
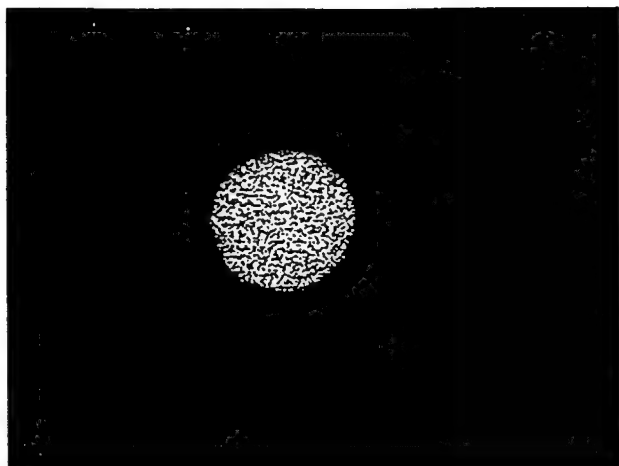


Fig. 3

# Agar degrading bacteria - a small study

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Abstract. Spheric macroscopically visible vacuoles were observed to develop 0.25% agar. The vacuoles were devoid of agar inside and enlarged until all agar had disappeared. The growth seemed independent of oxygen and the presence of sulfide or 3-mercaptopropionate. Microscopic examination revealed two morphotypes: long thin rods and shorter fat rods. Growth mode in different agar concentrations was examined by trapping streakings of inocula under glass slides and study colony development microscopically.

## Introduction.

Agar is produced from algae and is an extremely common material in microbiological laboratories. Agar is mostly expected to be an inert stable solid substrate for growth of microorganisms, but the degradation of agar is a well-known phenomenon (Reichenbach and Dworkin, 1981). That agar-like substances are present in nature from where they are extracted, makes such an utilization a probable, but mostly an unwelcome phenomenon.

## Materials and Methods.

Tubes: Originally for a different experiment, the following gradient tubes were made: Aliquots of 1 ml of 12.5% molten agar was tapped into 20 ml test tubes, which were placed in a 60°C water bath. Four ml of purple sulfur bacteria medium containing 5mM was added after being preheated for 10 min. in a 40°C water bath. The test tubes were then flushed with 80/20 N<sub>2</sub>/CO<sub>2</sub>, closed airtight and cooled to roomtemperature in a water bath so that a solid plug of 1.5% agar containing 4mM sulfide or MPA was formed. The 1.25% agar was placed in a 60° water bath while still hot after autoclaving. A similar medium but without sulfide or MPA was warmed up to 40°C in a water bath and mixed anaerobically with the 1.25% agar to yield a top agar with a concentration of 0.25%. Eight ml of this agar was then poured anaerobically on top of the plug in each of the test tubes to form a slush agar overlay. The test tubes were then left for three days to allow opposing gradients of oxygen and sulfide or MPA to establish through the top agar before inoculations. Nitrate gradient tubes were made by adding an anoxic 3 ml water phase containing 2 mM NaNO<sub>3</sub> after inoculation and subsequently flushing the headspace with 80/20 N<sub>2</sub>/CO<sub>2</sub> before closing the tubes airtight. That means that the top agar in the nitrate gradient tubes initially contained some oxygen as they were left for 3 days under atmospheric air.

The inoculum was sediment collected June 24 from Oyster Pond. Inoculations were made by filling approximately 0.2 ml of inoculum into a Pasteur pipette which had previously been pulled in a flame. The pipette was then inserted into the agar down to the top of the agar plug and retracted while slowly dispensing the inoculum in the entire length of the top agar.

Microscopic inspection of growth was performed by pulling a Pasteur pipette in a flame, sucking out material with it from discrete locations in the tube and examining it under the microscope.

Trapped colonies. In order to be able to inspect the growth of the colonies a setup for trapping colonies under cover-slips was made. Agar was washed in distilled water 4 times and made up in concentrations of 0.25, 0.75, and 1.5% in purple sulfur bacteria medium pH 7.2 without sulfide. This medium was used as it had already proved to sustain growth of the agar degraders in the tubes. A sterile microscopy slide was placed in a Petri dish in which was poured fifteen ml of 0.25, 0.75, or 1.5% agar. The agar covered the slide with an approximately 1 mm thick layer. Inocula were taken sterily from the colonies developing in the tubes and the number of cells was

counted in a counting chamber. On the basis of this count the inoculum was diluted down to 3000/0.1 ml and 30/0.1 ml. Plates were streaked with the diluted inoculum and a flame-sterilized 24×50 mm cover-slip was placed on the agar over the slide. The Petri dishes were incubated at 30°C in the dark. Growth was observed under a light microscope.

#### Results.

Growth in the tubes began as small ovoid spheres that grew larger until they merged with other spheres or touched the glass wall of the tube (Fig. 1). The interior of the spheres was a clear liquid. Growth continued until all slush agar had disappeared. The cells observed from the tubes were characteristic long slender rods. This corresponds well with the fact that *Cytophaga sp.* have been shown to be able to degrade agar (Reichenbach and Dworkin, 1981). The orientation of the cells seen under the microscope suggested that the cells grew very orderly side by side (data not shown).

Growth was observed in 0.75% agar where colonies enlarged their radius approx. 0.5 mm/day. The 0.25% agar turned out to be too thin to be stable. The 1.5% agar had some colonies, but the colony development was much slower than on the 0.75% agar. On the 0.75% agar two cell types were visible: 1) fat rods, that developed in tightly packed colonies which formed characteristic wave patterns (Fig. 2A) and 2) long slender rods that individually infiltrated the agar matrix. (Fig. 2B, C, and D). No clear zone without agar or cells was observed in any of the two types.

#### Discussion.

The characteristic pattern of spheres devoid of cells and agar in the tubes could not be repeated under the cover slips. This fact and the fact that the side-by-side growth pattern could not be observed under the cover slips suggest that either different organisms were observed in tubes versus under cover slips or that the state of the agar is very crucial in the development of growth. The fact that no growth of colonies was observed in the 0.25% agar and much more slow growth was observed in the 1.5% agar indicate that agar concentration is important.

The metabolism is most likely fermentation as the growth in tubes seemed independent of oxygen versus nitrate. Whether the nitrate tubes were completely anoxic is not clear though, but as they were tightly sealed and the headspace had been flushed with N<sub>2</sub>/CO<sub>2</sub> a small oxygen consumption should be able to remove all oxygen in a short time.

The setup of the cover slip covered agar should be improved to yield an even thinner agar layer, making microscopy better.

#### Literature:

Reichenbach, H. and M. Dworkin. 1981. The Order Cytophagales (with Addenda on the Genera *Herpetosiphon*, *Saprospira*, and *Flexithrix*). In: Starr, M.P. et al. (eds): The Prokaryotes. 1. Ed. Springer Verlag Berlin. 1981.

#### Legend:

Fig. 1. Growth of agar degraders in gradient tubes. The growth shows as spheres that merge or disappear upon touching the glass wall of the tube.

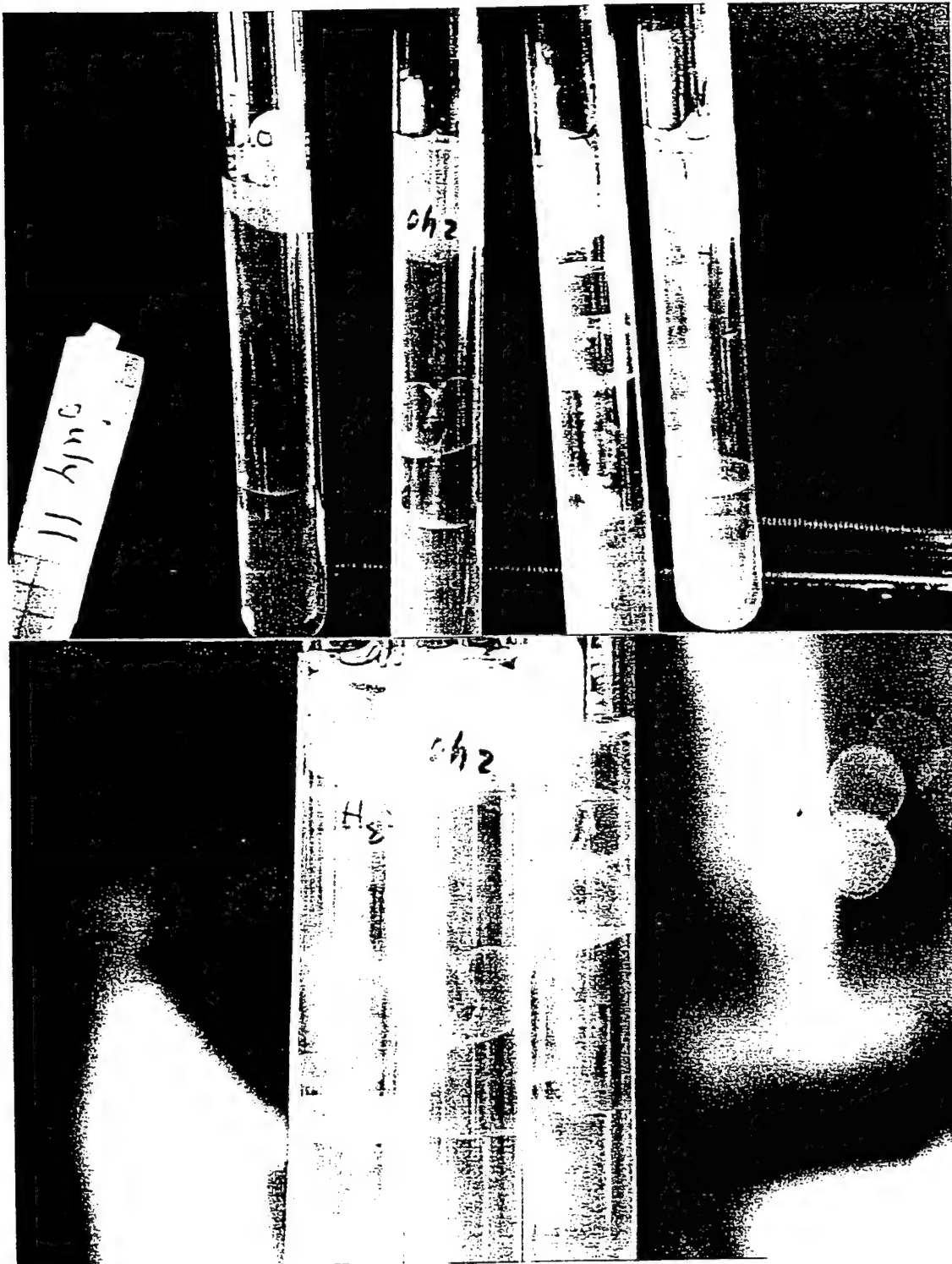
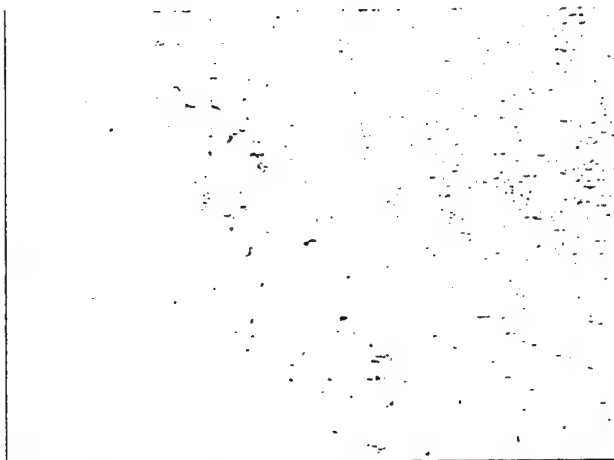
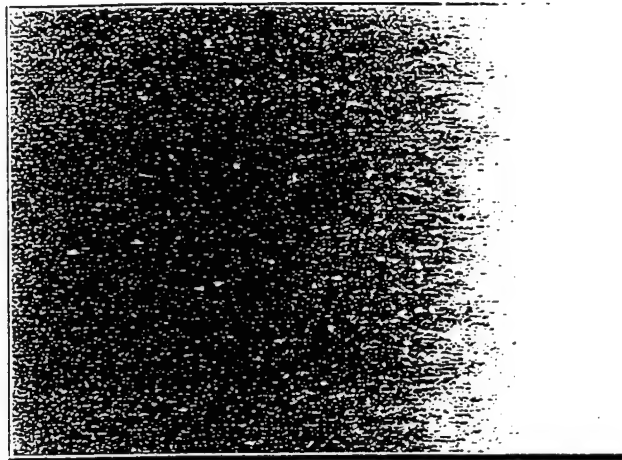


Fig 1

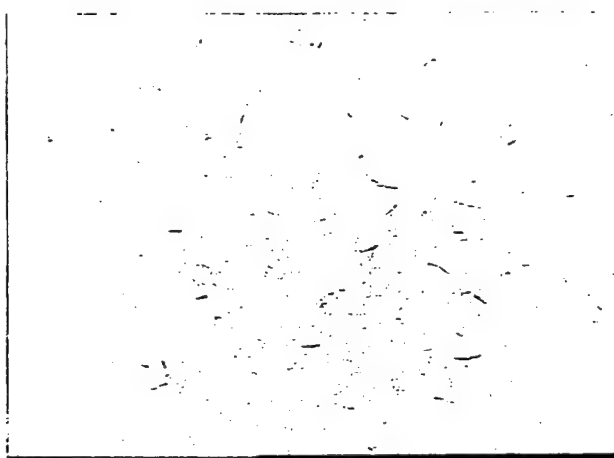
A



B



C



D

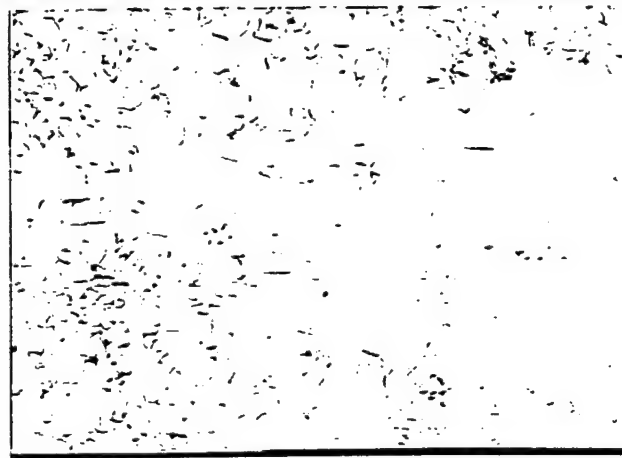


Fig 2

# MICROBIAL REDUCTION OF PHOSPHATE?

Microbial Diversity project, 1995

by

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## Introduction

Phosphorus is found in all parts of the biosphere and occurs most commonly in the form of phosphates in which the P-atom has an oxidation state of +5. Though phosphorus can exist in a range of oxidation states from +5 to -3, it is generally observed that it remains in the +5 state in natural transformations (Fenchel and Blackburn 1979). The biological phosphorus cycle thus appears to be relatively simple, lacking both a gaseous phase and the reduced oxidation states found in the nitrogen and sulphur cycles.

There are, however, numerous reports of trace levels of a reduced form of phosphorus, phosphine (PH<sub>3</sub>), being present in certain environments. The Will-O' the Wisp observed at night-time over peat bogs, swamps, cemeteries, recent battle fields and stagnant waters may not in fact be due to manifestations of the supernatural, but to the spontaneous ignition of gas containing traces of phosphine which evolved during the decomposition of animals in damp soils (Mellor 1940). In the 1920s a debate raged about whether the biological reduction of phosphate really occurred (Rudakov 1927, Rudakov 1929, Liebert 1927) and this argument has not yet been resolved.

A convincing demonstration of the prevalence of biological phosphine production was given in 1988 (Devai 1988). Examining the gases released from a sewage treatment works he found that 9-20% of phosphorus entering the system was converted to phosphine. He confirmed these observations using bacterial enrichments. Analyzing surface sediments of Hamburg harbour (Gassman and Schorn 1993) phosphine was found in the range of 1.2-56.6ng/kg. However, in 1972 Burford and Bremner failed to find any evidence for evolution of phosphine through microbial reduction of phosphate in waterlogged soils. In summary, it seems clear that trace levels of phosphine are undoubtedly present in the environment but the mechanism/organism responsible has not been found.

Thermodynamic calculations can be made to predict whether phosphate reduction is an energetically feasible reaction but even these seem to lead to no firm conclusion. Rudakov (1929) and Tsubota (1959) both showed that it was thermodynamically realistic, whereas Liebert (1929) disagreed. Devai (1988) stated that the formation of phosphine "cannot be ruled out or explained by the properties of redox systems".



There is also evidence that phosphine may play a role in anaerobic steel corrosion. Iverson (1968, 1984) noted that the corrosion product  $\text{Fe}_2\text{P}$  was evolved when phosphate was present in a steel corrosion experiment, and he suggested that this was due to the microbial production of phosphine which then reacts with ferrous iron to form  $\text{Fe}_2\text{P}$ .

Phosphate is a limiting nutrient in many environments due to the fact that it forms many insoluble salts (Ehrlich 1981, Fenchel and Blackburn 1979). It is also a vital nutrient for all biochemical processes. Why then should bacteria employ it as an electron acceptor? In certain environments phosphate will be in great excess. For example, living tissue is a rich source of phosphates in the form of phospholipids, ATP and apatite. (Maybe this could explain why Will-O' the Wisp is seen emerging from graveyards and battlegrounds). There is also evidence of phosphine evolution from blood and gastric juices stored at around  $40^\circ\text{C}$  and during the putrefaction of proteins (Mellor 1940). Sewage sludge also presents a bacteria with a phosphate rich environment, not only from feces but from phosphate added to detergents. In such environments it may give certain bacteria an ecological advantage if it is able to reduce surplus phosphate. There is an extraordinary diversity in microbial diversity in microbial use of inorganic compounds as terminal electron acceptors in anaerobic respiration, from the common  $\text{Fe(III)}$  (Lovley and Phillips 1988) to the more exotic  $\text{As(V)}$  (Ahmann et al 1994) and  $\text{U(VI)}$  (Lovley et al 1991).

The aim of the project is to attempt to observe and quantify phosphate reduction in bacterial enrichments from a variety of environments, and if this is successful, to isolate and characterize the organisms responsible.

### *Thermodynamic predictions*

Calculations were made to see if the reduction of phosphate to phosphine was thermodynamically viable:



$\Delta\text{G}^\circ_f$	-243.5	-123.75	0	+3.2	-92.26	-56.69
(kJ/mol)						

The standard Gibbs free energy change = +93.9 kJ/mole

( $\Delta\text{G}^\circ_f$  values were obtained from the CRC Handbook for Chemistry and Physics).

The reaction does not appear to be a thermodynamically favourable one, and becomes less favourable as the pH increases. However, if the phosphine or carbon dioxide gases are removed from the system by flushing or through a syntrophic association and the pH is reduced then the reaction may be energetically viable.

Calculations could not be made for the energetics of phosphate reduction to orthophosphite or hypophosphite (phosphorus oxidation states +III and +I respectively) because the necessary thermodynamic data i.e. free energy of formation/electrode potential was not available.

## Materials and Methods

The exact biological mechanism (if one exists) of phosphate reduction has not been established. However, the scant literature reports insinuate that phosphine is evolved from anaerobic environments and that the sulphate reducing bacteria *Desulphovibrio desulfuricans* may be able to generate phosphine (Iverson and Olson, 1984). Therefore the general strategy employed to try to enrich for phosphate reducing bacteria was the same as that for the enrichments for sulphate reducing bacteria but with no sulphate ion to act as terminal electron acceptor. Preliminary thermodynamic calculations demonstrated that the reduction of phosphate to phosphine was more likely at low pH so the enrichments were carried out at pH 7 and 4.

### *Innocula*

Six different innocula were used in the initial enrichments.

- A: Raw sewage from the inlet pipe of the Falmouth sewage works (pH = 6.7)
- B: The sandy black sulphate reducing layer of a microbial mat from the Sippiwissit salt marsh (pH = 6.8)
- C: A mixture of the lab SRB enrichments (lactate as the electron donor).
- D: A mixture of the lab SRB enrichments (acetate as the electron donor).
- E: Thiodendren veiled mud from the Sippiwissit salt marsh (pH = 6.8).
- F: The black outer surface of a rusty iron nail washed up on Sippiwissit beach.

### *Enrichment on inorganic phosphate*

In this enrichment only phosphate was used as the sole electron acceptor, whilst the electron donor and the pH of the enrichment was varied. The enrichment media was adapted from that used to enrich for sulphate reducing bacteria. The following salts were dissolved in 1 litre of distilled water:

		MARINE MEDIUM
NH <sub>4</sub> Cl	0.25g	
KCl	0.5g	
CaCl <sub>2</sub> .2H <sub>2</sub> O	0.06g	
NaCl	20.0g	
MgCl <sub>2</sub> .6H <sub>2</sub> O	0.62g	

This solution was autoclaved and cooled under N<sub>2</sub>/CO<sub>2</sub> (80/20) and aliquots of each of the following solutions added:

1M NaHCO <sub>3</sub> (under 100% CO <sub>2</sub> )	30mls
1M Na <sub>2</sub> S	1ml
SL-12 six vitamin solution	1ml
Six vitamin solution	1ml
Vitamin B solution	1ml
1M K <sub>2</sub> HPO <sub>4</sub>	10ml
1M KH <sub>2</sub> PO <sub>4</sub>	10ml

This solution has a pH value of  $7 \pm 0.2$ . Half of this media was then dispensed into serum bottles under  $N_2/CO_2$  (80/20). The pH of the remaining solution was reduced to 4 using sterile HCl and was dispensed as above. Inoculum was added to the medium at a ratio of 1:100. For each inoculum at each pH, lactate, acetate, sodium sulphite or formate were added as possible electron donors to give a final concentration of 10mM.  $H_2$  was also added in gaseous form as a possible electron donor. In the enrichments with  $H_2$  and sulphite as the electron donors the gas phase of the media contained 10%  $CO_2$  to act as a carbon source. The enrichments were incubated at  $30^\circ C$ .

Secondary enrichments were made from the enrichments with the greatest growth. In this case controls were made for each pH with a) no electron donor b) no phosphate c) sterile inoculum (autoclaved sewage).

#### *In situ hybridization*

*In situ* hybridization was carried out on enrichments from black sulphate reducing mud (enrichments B) at pH 7 and 4 that had lactate as the electron donor. The aim of this was to attempt to identify which types of organism had been enriched, and to compare any differences between bacteria observed at pH7 and pH4.

The method followed was that of Bauer (lab exercise). Probes used were:

- a) SRB, Flavo,  $\alpha$ ,  $\beta$ ,  $\delta$  and universal probes for  $45^\circ C$  hybridization temperature.
- b)  $\gamma$  (enteric), Archea and universal probes for  $37^\circ C$  hybridization temperature.

#### *Bacterial Isolations*

Agar shake series were made according to the method of Jorg Overman for sulphate reducing bacteria but again with no sulphate present and the phosphate present at 20mM (see Marine medium above). Isolations were made from enrichments with the greatest growth, and from both pH7 and pH4 to enable a comparison to be made between bacteria that are capable of growing at these pH conditions. Isolations were made under the same conditions of pH/with the same electron donor as the enrichment from which the inoculum was derived. The tubes were incubated in the dark at  $30^\circ C$ .

#### *HPLC analysis for phosphate and phosphite*

Phosphate and phosphite were measured by HPLC (Waters) equipped with an anion exchange (IC PAK A HC) column eluted with gluconate/borate. The eluens was made up as follows: A stock solution X was made by dissolving 16g sodium gluconate, 18g boric acid, 25g sodium tetraborate decahydrate (Borax) and 250ml glycerol in 1litre of ultrapure water. The eluens was made by mixing 20ml stock X, 20ml 1N butanol and 120ml acetonitrile in a total of 1litre ultrapure water. The eluens was filtered through a  $0.22\mu m$  filter and degassed before use. The flow rate of the HPLC was 2ml/minute. The detector was a 430 Waters conductivity meter running at  $35^\circ C$ .

Samples selected for HPLC phosphate analysis were B enrichment with electron donor source of lactate or acetate and E enrichment with lactate as the electron donor. Of all the enrichments these demonstrated the greatest growth. The 2 negative controls in this experiment were the A enrichment with lactate (which showed very poor growth) and the E enrichment with formate with sustained no growth at all.

#### *Enrichment on organic phosphate*

The same method was employed as for enrichment on inorganic phosphate but in this case raw sewage was the only inoculum used and only organic phosphate sources were added to the enrichments. Phytic acid or phosphatidyl choline (dissolved in reagent grade alcohol) were added to the medium to a final concentration of 10mM. The sodium chloride concentration in the medium was reduced to 1g/l NaCl.

#### *The ultimate experiment*

Phosphine is "spontaneously flammable in air if there is a trace of  $P_2H_4$  present and burns with a luminous flame. It combines violently with oxygen" (Merck index). Therefore, given that the ideal analytical tool, a GC method to detect phosphine gas, was not available, the ultimate experiment in this project was to attempt to ignite the gases that may have been evolved during the enrichment procedure. This was done by opening the serum bottles and waving the neck across a flame.

## RESULTS AND DISCUSSION

### *Enrichment on inorganic phosphate*

After 8 days the enrichments were analyzed for turbidity. The most turbid enrichments were those derived from innoculum B, the black sulphate reducing layer from Sippiwissitt salt marsh. No growth was observed in the enrichments with the sewage innoculum, and only a black precipitate (with no bacterial growth) was observed in the enrichment from innoculum F, the rusty nail. The enrichments using sulphate-reducing mud sample E showed a little growth. Table 1 below shows the levels of growth observed in the B enrichments.

pH	Electron donor	Growth
7	Lactate	+
7	Acetate	+
7	Hydrogen	-
7	Sulphite	-
7	Formate	-
4	Lactate	++ (spirillum)
4	Acetate	+
4	Hydrogen	-
4	Sulphite	+
4	Formate	+

' + ' = growth, ' - ' = no growth

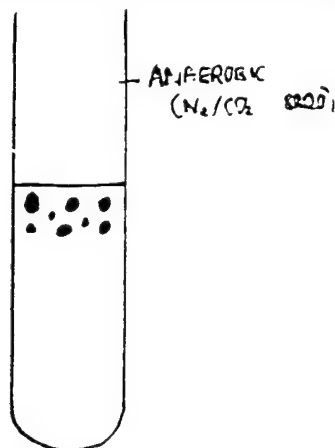
*Table 1. Growth in primary enrichments for phosphate reducing bacteria with SRB mat black layer as the innoculum.*

It should be noted that this is a relative scale and compared to a culture of say *E. Coli* on nutrient broth, the culture was very thin, with most of the turbidity derived from precipitation of inorganic material. The bacteria present were all small rods or cocci, but spirillum were observed in the enrichment at pH 4 with lactate as the electron donor (indicating that they may not be as fragile as they often appear to be). The enrichment procedure is selective *ie* growth is not observed in every bottle. There is more growth at low pH and acetate or lactate seems to be the preferred electron donors.

Secondary enrichment from B primary enrichments did not have time to fully develop but at the time of writing the only enrichment that had grown at pH 7 was that with acetate as an electron donor. The controls (with either no electron donor, no phosphate or with sterile innoculum) did not sustain growth. Secondary enrichments at pH 4 showed a small amount of growth with lactate and sulphite as electron donors, but growth was observed in the control with no electron donor added.

### *Bacterial isolations*

After an incubation period of 7 days growth could only be observed in medium of pH 7 (from enrichments of pH 7) at dilutions of  $10^{-1}$  to  $10^{-3}$ . The round white colonies were only present at the top centimetre of the agar (Fig.3).



*Figure 3. Colony formation in the agar shake tubes at pH 7.*

Some colonies were noticeably compact whereas others were more diffuse. The 2 colony morphotypes were analysed under the microscope (Fig. 4).



Compact white colonies:  
'Pure' culture of long rods

Diffuse colonies:  
Long rods and highly motile cocci

*Figure 4. Composition of the colonies derived from the agar shake series.*

Time constraints did not allow further analysis of these cultures.

After incubating for 13 days the other enrichments were studied under the microscope to establish whether the turbidity that was often present was due to precipitation of some the inorganic components of the medium or due to bacterial growth. The following observations were made:

Enrichments A (sewage inoculum)

Small amounts of growth were observed at both pH 4 and 7.

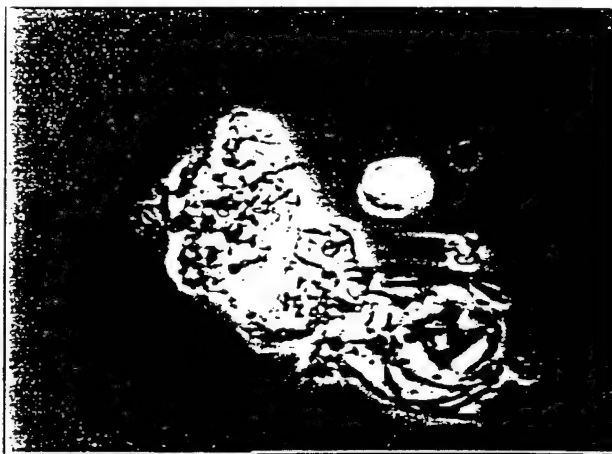
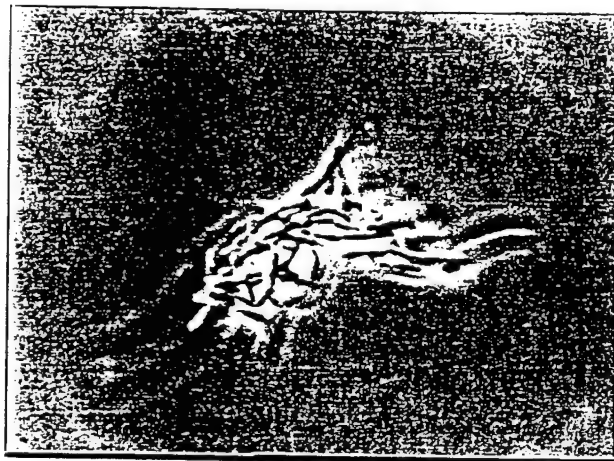
Enrichments C and D (lab collection of SRB enrichments as innoculum)

Trace amounts of growth were observed with lactate as the electron donor, but there was no growth with acetate as the electron donor.

Enrichments E (SRB mud as innoculum)

pH	Electron donor	Observation
4	Lactate	++ Rods and cocci. Long filamentous organisms wrapped around sediment (figure 1)
4	Acetate	-
4	Hydrogen	++ Short rods and cocc.
4	Sulphite	-
4	Formate	-
7	Lactate	++ Rods and cocci
7	Acetate	-
7	Hydrogen	-
7	Sulphite	++ Rods & cocci & the occasional long spirochete
7	Formate	-

Table 2. Growth in enrichments E (with SRB mud as the innoculum).



Microscopic analysis of these enrichments did not detect any signs of life. The turbidity present in the flasks was due solely to precipitation.

#### *HPLC analysis for phosphate and phosphite*

Phosphate (in the form of  $\text{NaH}_2\text{PO}_4$  pH7) was easily and accurately determined by this method. However no hypophosphite could be detected (I used standards of low concentration up to  $100\mu\text{M}$  because I predicted that if any phosphate had been reduced, at this early stage in the enrichment only low levels of the reduced form would be found). Levels of phosphate measured in certain enrichments are shown below in Table 3:

Sample	Growth?	[Phosphate] mM
B lactate pH 7 enrichment	+	19.46
B acetate pH 7 enrichment	+	22.68
E lactate pH 7 enrichment	+	22.26
A lactate pH 7 enrichment	trace	22.33
E formate pH 7 enrichment	-	15.54

*Table 3. Phosphate concentrations of selected enrichments after X days on incubation.*

Phosphate levels do not decrease in proportion to the amount of growth observed. The large discrepancies in the concentration of phosphate measured can be explained in terms of the amount of phosphate-containing precipitate forming in a given enrichment. Phosphate readily forms insoluble precipitates with calcium and magnesium even when these ions are present in very low levels. This precipitation renders the phosphate undetectable in the aqueous phase of the enrichment from which the sample for HPLC analysis is derived. Given the small amount of bacterial growth in the enrichments, it is likely that the dominant factor governing the amount of phosphate measured is precipitation of the phosphate rather than biological processing.

The value of a measuring the amounts of phosphate used up in the enrichments as an indication of phosphate reduction is limited because it is difficult to measure of how much phosphate the cells would be using in 'normal daily life', not as an electron acceptor. Ideally the method would be applied to the secondary enrichments which would have had less carry-over of nutrients and for which suitable controls were available for comparison. However, not enough growth was observed in these enrichments to be able to observe significant decreases in the concentration of phosphate.

#### *The ultimate experiment*

Unfortunately (or fortunately for some) this experiment did not yield explosive results, or even one little luminous flame! 3 cultures seemed to make the flame go "phut" a tiny bit, but that could have just been due to air currents as the gas phase emerged from the serum flask or wishful thinking on my part.



### *In situ hybridization*

The bacteria present in the enrichment at pH 4 and pH 7 tested positive with only the SRB probe and the universal probes. The bacteria present in these enrichments are predominantly sulphate reducing bacteria (this is hardly surprising given that the inoculum for the enrichment was sulphate reducing mud). As can be seen in fig. 2 some cells fluoresced brighter than others but this was also true of the universal probe too. There was no observable difference between the bacteria from the enrichment at pH 4 and pH 7. The cells were mostly small rods and cocci which were very skinny compared to the *Rhodospirillum centenum* used as a positive control organism.

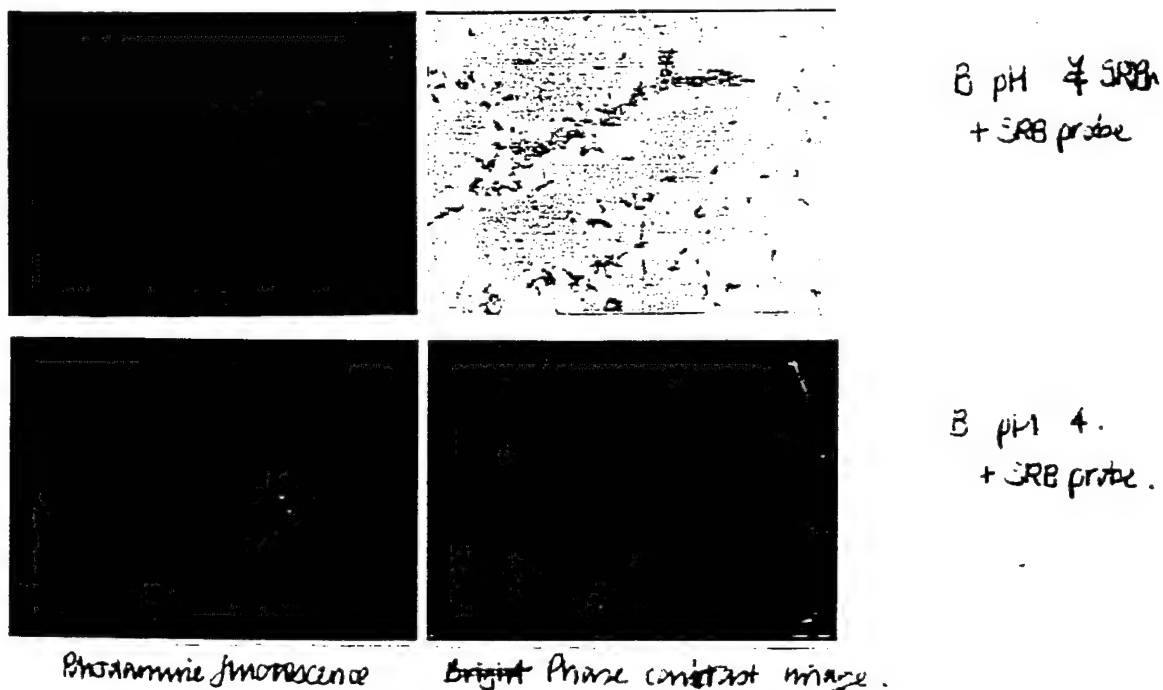


Figure 2. Hybridization probe results

### *Enrichment on organic phosphate*

With phytic acid as a phosphate source growth was observed in all the enrichments, including, unfortunately, the controls (except for the one with sterile inoculum). It thus appeared that organisms within these enrichments were capable of growing at the expense of substrates other than those intended. Again, significant growth was observed at pH 4 and 7, and spirochetes were observed in the enrichment at pH 7 with lactate as the electron donor.

Phospholipid, when added to enrichment medium, formed a milky white emulsion. It was therefore difficult to assess by eye whether bacterial growth had occurred in these enrichments. Microscopic analysis revealed that any turbidity was due to lipid emulsion not to bacterial growth.

## Conclusions

The results shown above neither confirm or rule out the possibility of biological phosphate reduction. They do however nicely demonstrate that bacteria were surviving/growing under very stringent conditions in terms of their choice of electron donor/acceptor and in terms of the pH in which they were placed. Given that estuarine bacteria require up to a week to switch on the necessary enzymic mechanisms and to grow sufficiently to cause a significant decrease in levels of the highly biodegradable surfactant SDS, it seems very unlikely that I would have observed a significant reduction in the levels of phosphate in the enrichments given the limited time scale of the project.

It is quite reassuring to read that Devai (1988) had to wait 56 days before he could observe significant reductions in the phosphate levels in his *in vitro* experiments to detect phosphate reduction. However, the lack of further publications from him on this topic would seem to indicate that maybe his enrichments for phosphate-reducing bacteria were not so successful after all.

Though phosphine is undoubtedly present in trace quantities in certain environments, the mechanism for its production remains a mystery. The secret of Will-O'the Wisp lives on.

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## Microbial Diversity Personal Project

# Isolation of Cellulolytic Bacterium using Turnip as Cellulose Source (Substrate)

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## Introduction

Interested in cellulose decomposition and in the microorganisms that produce the relevant enzyme systems has been stimulated by a desire for a greater understanding of one of the most important processes in nature. This, in turn, has been prompted by the need to optimize exploitation of the potential of cellulose as a source of energy and chemical feedstocks and to improve the efficiencies of digestion of fodder by ruminants.

Cellulose accounts, on average, for 50% of the dry weight of plant biomass, it also accounts for about 50% of the dry weight of secondary source of biomass, such as the surpluses, wastes of agricultural, forest, industrial and domestic origin. Cellulose is a homopolymer consisting of glucose units joined by  $\beta$ -1,4 bonds. The disaccharide cellobiose is regarded as the repeating unit in cellulose in as much as each glucose units is rotated by  $180^\circ$  relative to its neighbor.

Cellulolytic microorganisms are ubiquitous in nature, and representative species are found in many different generation and in a great variety of environments such as soils, swamps, seawater sediments, cotton bales, animal gut ect. Many enrichments have been done for cellulolytic bacteria and many different types of cellulolytic bacteria have been isolated and pure cultured. All of these, enrichments have used microcrystalline cellulose, carboxymethyl cellulose (CMC) or other forms of purified cellulose as carbon sources. These celluloses are quite different from the cellulose found in many vegetables or other natural sources. In that natural, cellulose is more hydrated than purified cellulose. As turnips contain a high concentration of hydrated cellulose, an enrichment using turnip slices as the cellulose source could turn up with new type of cellulose-degrading bacteria.

In this project, I am using boiled turnip mash as a substrate to isolate cellulolytic bacteria. Congo Red stain is used to indicate cellulose hydrolysis, Cellulose Azure is used as substrate to do cellulose assay.

## Materials and Methods

**Enrichment, Culture conditions and media:** Enrichment: The sources of bacteria were the gut of a terrestrial snail found in Dr. E. Leadbetter's backyard and Eel Pond sediment. Boiled and autoclaved turnip slices was used as carbon source.

Media: All media contained ( g / l )

K <sub>2</sub> HPO <sub>4</sub>	1
NH <sub>4</sub> Cl	1
MgSO <sub>4</sub> · 7H <sub>2</sub> O	0.05
CaCl <sub>2</sub> · 2H <sub>2</sub> O	0.02

add water to 1000 ml.

In enrichment broth, turnip slices were made by boiling 3 times and then autoclaved; in the isolation media, turnip paste were made by boiling turnip slices 3 ~ 5 times, throw the water each time, then use blend mash the turnip into very fine paste solution; Washed Agar were made by Add 25 g Agar to 5 l distilled water, stirred for 1 hour then replace water for one cycle, generally 5 ~ 6 cycles, original 25 g agar used for 1000 ml media. Cellobiose (2 mg / ml final concentration), cellulose (ball milled filter paper, 1 mg / ml), CMC ( 1 mg /ml) and xylan (1 mg / ml) agar plate were also made by adding these sugars to the base media given above. Cellobiose, glucose and CMC broth media were made by adding those sugar solution to the autoclaved base media before inoculation. 10% cellobiose and glucose stock solutions were filter sterilized

Culture Conditions: Aerobic isolates were cultured in 37°C incubator. Anaerobic isolate was culture in room temperature under N<sub>2</sub> + CO<sub>2</sub> in the anaerobic chamber.

**Congo Red test:** Follow protocol according to Teather, after appropriate growth period, flood the plate with the Congo Red Solution ( 1 mg / ml in water) for 15 minutes. For CMC, pour off Congo Red and flood plate with 1 M NaCl for 15 minutes. Visualized zones of hydrolysis can be stabilized for at least 2 weeks by flooding the agar with 1 M HCl, which will change the dye color to blue and inhibit further enzyme activity.

**Oxidase test:** Use spot test oxidase reagent form Difco lab. Pick up colonies from plate, put them on the filter paper, drop one or two drops of oxidase reagent to the colonies, check blue color for positive result after 5 minutes. Colorless is negative.

**Electron microscopy:** Cell were negatively stained for microscopy. Carbon coated grids were used to absorb cells from well prepared bacterial cell suspension for 2 minutes. Uranyl acetate solution ( 1% wt / vol, pH 4.5) was used. Negatively stained preparations was examined using a Zeiss 10CA transmission electron microscope.

**In Situ 16s RNA Hybridization:** Follow Dr. Sandra A. Nierzwicki - Bauer's Handout.

**Cellulose Assay:** Use cellulose Azure as a substrate. Suspend 100 mg of cellulose Azure in 10ml phosphate buffer with no cellulose. In microcentrifuge tube, put 1 ml cellulose Azure solution and 1 ml cell culture incubate for different period time at room temperature. Centrifuge for 3 ~ 4 minutes, measure supernatant absorption at 570 nm, DH<sub>2</sub>O and cellulose were used as negative and positive controls.

## Results and Discussions

**Enrichment and morphology** Enrichments were streaked on the isolation plate put under aerobic and anaerobic conditions. there were on growth from sea sediment. From snail gut, three aerobic and one anaerobic strain were isolated

### Colony and Morphology

isolates	colonies	morphology	congo red test	oxidase test	Gram stain	aerobic or anaerobic
IS1	large white, very convex	single, thin but long cells, terminal endospore former	+	-	+	anaerobic
IS2	white, flat, irregular edge, look like mycelial colonies	large rod form filaments, middle endospore former, gliding	+	+	- (?)	aerobic
IS3	tiny, white, convex	rod or cocci, nonmotile, single or pair, nonendospore former	-	-	-	aerobic
IS4	large white glistening, regular edge	short rod, motile, single no endospore	-	-	-	aerobic

From the turnip plate, IS1 and IS2 gave clear zones around the colonies, so this two strains were chosen to do further studies.

**Physiology** Inoculate IS1 and IS2 on different plates which contain different carbon source

	CMC	cellobios	cellulose	xylan	unwashed agar	washed agar
IS1	++	++	+	+	+	-
IS2	++	+	-	+	+	-

++: grow very well

+: grow well

-: no growth

IS2 was taken from plate and inoculated to liquid broth medium under room temperature, and there were no growth. But if add 0.01% yeast extract, all of them grow rapidly, the negative control which has no carbon source also grow, but not as turbid as other tubes with carbon source in it. So IS2 may need some growth factor from yeast extract.

### 16s ribosome RNA hybridization and cellulase assay

12 different 16 rRNA probes were used to do the hybridization. For the anaerobic isolation IS1, all the probes are negative, even universal probe. the reason could be due to the old culture which contain all endospores instead of vegetative cells. It's hard for the probes to

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get into the cell and gave the signals. For IS2, prime A which is universal probe, was the only positive result. maybe this organism is beyond the detect range of all probes. Besides, there are some immature aspects of 16s rRNA techniques

Only IS2 was performed of cellulase assay. the mechanism for this assay is cellulose azure which is cellulose derivative that has azure binding to cellulose monomer, when cellulose azure is degraded by cellulase, the product show optical absorption at 570nm. This assay took very long time, the positive control turn pink after 4 days (86 hours). the assay was carried out in room temprature, the positive controwas made by adding 25 mg purified cellulase to 1 ml phosphate buffer. Distilled water was used as negtive control, the sample is more like negtive control. overall, this assay was a very rough cellulase assay. And more this assay was designed for complete cellulase system. IS2 probably don't possese a complete cellulase system since it didn't grow on cellulase plate, but it can use cellobiose and CMC, so it may produce endoglucanases or  $\beta$ -glucosidases, not the complete system, those bacteria, as suggested by Beguin, called "pseudocellulolytic" in contrast to few bacteria that synthesize the complete enzyme system that could result in extensive hydrolysis of the crystalline material found in nature, which is called "true cellulolytic". It is possible that IS1 could be "true cellulolytic" because it grow on cellulose plate. Further studies have to be done to define this bacterium.

**Acknowledgment:** I thank Dr. A Salyers for her direction and disscusion, and Jie Huang for typing and encouragement

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## Microbial Diversity Personal Project

# Isolation and Characterization of Anaerobic Cellulolytic Fungi from Anaerobic Environment

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## Introduction

Cellulose is the most abundantly produced biopolymer in terrestrial environment. Each year photosynthetic fixation of  $\text{CO}_2$  yields more than  $10^{11}$  tons of dry plant material worldwide and almost half of the material consists of cellulose. Another major source of cellulose is municipal wastes.

Most Cellulose is degraded aerobically, but 5 - 10% is degraded anaerobically. Thus, vast quantities of cellulose are degraded by cellulose- fermenting microorganisms in habitats anaerobic. In the past fifty years, aerobic cellulolytic bacteria have been intensively studied, including thermophilic and mesophilic fungi. By contrast, anaerobic fungi have been ignored for a long time and were first discovered in 1976. They were found in the rumen and hindgut of herbivores. They have a nice developmental cycle, that is observable in vitro and they possess cellulolytic and other hydrolytic capabilities.

Anaerobic fungi isolated from ruminants have been studied extensively (such as *Neocallimastix* spp.). In contrast to strains isolated from non-ruminants (such as *Piromyces* spp.). Anaerobic fungi isolated from ruminant and non ruminant herbivores have a high digestion capacity for cellulose (Teunissen et al. 1991). Species isolated from ruminants have been shown to produce extracellular enzymes such as exoglucanase, endoglucanase, B-glucosidase and xylanase activities in vitro, when grown on cellulose. Production of the enzymes is often associated with the ability to produce an exoglucanase, which acts as a cellobiohydrolase. Fungi from the rumen such as *Neocallimastix* species have been shown to digest cellulose with rates varying between  $0.04$  and  $0.06 \text{ g/l h}^{-1}$  (Bauchop and Mountfort 1981; Lowe et al. 1987 b; Phillips and Gordon 1989). *Piromyces* species, isolated from non-ruminants, could digest cellulose at a rate comparable to *Neocallimastix* spp.

Although free-living anaerobic cellulolytic fungi similar to those found in the rumen (124) have not yet been found, Durrant et al reported isolating, using anaerobic culture conditions, two strains of morphologically and physiologically distinct cellulose - fermenting fungi from soil. Both strains grow and utilize cellulose more rapidly when incubated under microaerophilic conditions, and one strain degrades cellulose most rapidly in well-aerated culture. Active cellulose and xylanase systems are produced by both strains, and enzymes are present in culture supernatant fluids. Clearly, fungi may play a significant role in the anaerobic degradation of cellulose in soils and sediments, and further studies are needed to explore their potential contributions.

The goal of my project was to isolate anaerobic cellulolytic fungi from different anaerobic environments such as the termite gut and Cedar swamp sediments. I am going to look at

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cellulose activity, fermentation end product (possibly pathway!) as well as their interaction with methanogens by  $H_2$  transfer!

## Methods:

**Chemicals and solid substrates.** All chemicals were of Analar grade and unless stated otherwise were made up in distilled water. All vitamins and antibiotics were obtained from Sigma. Whatman no. 1 filter paper was used for cellulose substrate and was treated with phosphoric acid (Wood, 1971) and then pebble milled for 4 d at 4°C.

**Preparation of media.** Media were prepared, stored and inoculated using the aseptic, anaerobic techniques of Hungate (1950), Bryant (1972) and Miller & Wolin (1974). Except for the reducing agent solution (prepared and dispensed under oxygen-free  $N_2$ ), liquid media and solutions used to prepare media were dispensed under oxygen-free  $CO_2$ . Solid media were prepared by adding agar (final medium concentration, 18 g/l) to basal solution.

**Medium A.** Medium A. contained, per litre: basal solution A, 830ml; water (when insoluble substrates were used) or glucose solution (37.5g/l), 100 ml;  $Na_2CO_3$  solution (80g/l), 50 ml; vitamin solution, 10ml; and reducing agent solution, 10 ml. Sometimes 60 ml of the water was replaced with 50 ml antibiotic solution and 10 ml lysozyme solution.

Base solution A contained: yeast extract, 2g; Trypticase peptone, 2g; clarified rumen fluid (Bryant & Robinson, 1961), 150ml; mineral salts solution [as described by Leedle & Hespell (1980) except that the nitrogen source was 0.54g  $NH_4Cl$ /l], 75 ml;  $K_2HPO_4$  solution (6g/l; Leedle & Hespell, 1980), 75 ml; haemin solution, 10ml; fatty acid solution, 10ml; resazurin solution (1g/l), 1 ml. The pH of basal solution A was adjusted to 6.8 with 1 M-KOH and the volume was made up to 830 ml with water or a suspension of cellulose (final medium concentration 10 g/l for the cellulose overlay plates. Haemin solution was prepared by dissolving 0.1 g haemin in 10 ml ethanol and adjusting the volume to 1 litre with 0.05 M-NaOH. The fatty acid solution was prepared by mixing 6.85 ml acetic acid, 3.0 ml propionic acid, 1.84 ml butyric acid, 0.55 ml 2-methylbutyric, 0.47 ml isobutyric acid, 0.55 ml valeric acid, and 0.55 ml isovaleric acid with 700 ml 0.2 M-NaOH. The pH of the fatty acid mixture was adjusted to 7.5 with 1 M-NaOH and its volume was adjusted to 1 litre with water.

Vitamin solution was prepared in 5mM-HEPES buffer and contained (g/l): 1,4-naphthoquinone, 0.25; calcium D-pantothenate, 0.2; nicotinamide, 0.2; riboflavin, 0.2; thiamin. HCl, 0.2; pyridoxine.HCl 0.2; biotin, 0.025; folic acid 0.025; cyanocobalamin, 0.025; and p-aminobenzoic acid, 0.025. The reducing agent solution contained 2.5 g  $Na_2S$ , 0.9  $H_2O$  and 2.5 g L-cysteine.HCl in 100 ml water. The antibiotic solution contained (g l<sup>-1</sup>): streptomycin sulphate, 2; penicillin G, 8; chloramphenicol, 6; oxytetracycline, 5; neomycin sulphate, 6. The lysozyme solution contained lysozyme (4 g /l) plus EDTA (disodium salt; 3 g/l).

Basal solution A,  $Na_2CO_3$ , glucose, water and the reducing agent solution were pre-reduced (boiled and gassed with oxygen-free  $CO_2$ ).

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**Sterilization and preparation of liquid and solid media:** The Antibiotic, lysozyme, vitamin, Rumen fluid and 10% cellulose solutions were sterilized by membrane filtration (0.22  $\mu$ m pore diameter). All other solutions and Agar media were autoclaved at 121°C for 20 minutes. Liquid media were dispensed in 3.75 ml volumes in thick-walled glass tubes. Cellulose medium was stirred when dispensed in order to suspend cellulose fiber as even as possible. After autoclave and before inoculation following solutions were added to each tube separately!

	volume ml / tube
Rumen fluid	0.75
Antibio Sol.	0.25
lysozyme + EDTA Sol.	0.1
cellulose	0.15

For the roll tube media, first melt the Agar media in boiling water, then put into 45°C water bath, every solution was added under N<sub>2</sub> flow using Hungate techniques

**Samples and environment culture:** Three different samples were taken from different source. Fresh termite gut. Volta flame Pond sediment, Cow Rumen. First have very heavy inoculation from sample to cellobiose broth media, then inoculate 1 ml to each of tubes of cellulose and cellobiose agar and broth media. For the roll tubes, as soon as inoculation, put the tubes into ice, roll it horizontally until media solidified.

Enrichments of termite gut & sediment were incubated at 30°C incubator, cow fluid samples was incubated at 37°C. Roll tubes were incubated vertically and all broth tubes were incubated horizontally.

## Result and Discussion:

After 5 days incubation, yeast cells (only guess, due to big, budding cells) were found in the cellulose broth enrichment. Some filamentous endospore former were also found in the culture from microscope. There are lots of cells attached to the filaments. Those cells could be methanogen, because bacterial antibiotics don't inhibit methanogen growth, and those methanogen could use H<sub>2</sub> released from fungi fermentation. This is also why we incubate the broth culture horizontally. In the sediment and cow fluid broth enrichment, there were no growth, no filament or even no cells found. All roll tubes had no growth or visible colonies. The possible reason for this phenomenon could be: Termite gut and sediment: 1) there are no anaerobic cellulolytic inhabited in termite gut, or even some, they are extremely hard to grow *in vitro*. 2) They don't like the media which provides a rumen-like environment. 3) Exposed to air! Cow Rumen fluid: The sample was taken 2 days before enrichment, and stored a long time in the fridge, this could be fatal to rumen fungi, because they really don't like low temperature. Generally, the rumen digesta were collected by suction into CO<sub>2</sub> filled tyermos flasks. Then enriched with milled barhey straw, the substrate could be another important reason. They probably prefer to use barhey straw other than pretty pure cellulose paper.

Morphologically, anaerobic fungi could be single cells. So the big budding cells could be another species of fungi. The possibility has to be further tested and those single cells cellulolytic ability needs to be characteristicized!

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**Acknowledgment:** I thank Joel for his direction and discussion, and Jie Huang for her typing and encouragement.

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Partial stratification *in vitro* of microbial subpopulations from the Salt Marsh  
Microbial Mat, Sippewissett MA.

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Microbial Diversity Course

Summer 1995

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# Partial stratification *in vitro* of microbial subpopulations from the Salt Marsh Microbial Mat, Sippewissett MA.

Elena Hilario

## Introduction

Microbial mats are stratified communities of bacteria and few eukaryotic organisms, all closely related at the metabolic level. The community is based on very well defined gradients that support different living forms at different depths. There are several types of microbial mats: hot springs microbial mats, where purple sulfur bacteria and gliding bacteria live at temperatures ranging from 40 up to 70°C, approximately. In non-thermal environments, masses of photosynthetic bacteria (cyanobacteria, purple sulfur and non sulfur bacteria, green sulfur bacteria and sulfate reducers) bloom at intertidal zones in salt marshes. This second type of microbial mat has been extensively studied on the past years due to its ecological, taxonomical, biochemical (photosynthetic mechanisms, mainly) and evolutionary relevance.

Although great efforts have been made to isolate the organisms present in microbial mats, only the most represented or easy-to-cultivate ones have been identified. It has been estimated that they could represent about 5% of the whole community. New efforts and probably radically different methods of isolation have to be considered for future studies.

The development of microbial model systems under controlled laboratory conditions has been studied for relatively simple communities such as co-cultures or single species systems. There are several types of laboratory model systems: open model systems (multistage chemostats, gradostats, gradostats with cell separation), and closed model systems (batch cultures, gel stabilized systems) and biofilms.

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Gel stabilized systems are characterized by well defined boundaries: carbon source, proton and gas concentrations, etc. An interface develops when countergradients meet and a microenvironment is developed. Organisms capable of tolerate such conditions overgrow in this zone. After a while, a community becomes spatially ordered if solutes deep diffusing.

Model systems have been heavily criticized because they could oversimplify the natural environment, but their development and study should not be dismissed taking into account that direct field studies, in some cases, are close to impossible. We should also keep in mind that model systems for mat communities could have left aside key organisms unable to adjust to the new semi-artificial environments, as well as non-bacterial members such as bacteriophages and lower eukaryotes which could be playing very important roles in genetic information exchange and population control. Nevertheless, model systems for microbial communities can expand our knowledge of the complex relationships among their residents.

The present report includes experiments done in order to observe how could a microbial community spatially organize itself under laboratory conditions. The whole microbial community of the salt marsh in Sippewissett MA, was used as inoculum in semisolid sea water agar and poured on top of an agar slant containing different carbon sources. Different light conditions were also tested. All physical conditions and chemical composition of culture medium were kept as simple as possible.

## **Materials and Methods**

A mixed sample (~30 ml) of the salt marsh microbial mat from Sippewissett MA was resuspended to a final volume of 45 ml with sterile and filtrated sea water. The sandy sample was shaken vigorously for 10 min and let it stand at room temperature for 15 min

until a particle-free supernatant was obvious. The supernatant containing a mixed bacterial population among some other organisms (ciliates, nematodes, etc.) was diluted 1:10, 1:50 and 1:100 with sterile and filtrated sea water. Equal volumes of diluted samples were mixed with filtrated sterile sea water-agar 1.5% (final concentration 0.75% agar, in a final volume of 5 ml). The semisolid agar-sample mixture was poured into two different sets of carbon source-agar tubes: a) yeast extract 0.25%, filtrated sea water and agar 1.5%; or b) sterile liquid phase of boiled mud obtained from the salt marsh mixed with agar 1.5% in filtrated sea water. At least five tubes for each set of conditions were poured. The carbon source was poured in two different ways: as a plug at the bottom of the test tube, or as a gradient in tilted test tubes (slants). Once the semisolid agar was poured, the tubes were cooled down immediately on ice to prevent diffusion or local accumulation of the inoculum. The agar tubes were overlayed with sterile sand and exposed to two different light conditions: a) direct sunlight; and b) buried up to the sand overlay in a wet sand bucket or beakers covered with clear plastic film or glass Petri dishes, according to the number of tubes prepared. It is important to keep the sand wet, not flooded, in order to maintain a moisturized environment and prevent the agar tubes from drying out. The buried tubes were covered with hydrated dialysis membrane (cut off 12 000-14 000 Da) to let the sunlight go into the test tubes. The tubes exposed to direct sunlight were covered with plastic closures only.

Both sets of tubes were exposed to sunlight, maintaining a day-night cycle according to the local weather oscillations. Temperatures during incubations range from 30 up to 45°C during day, and 18 to 20°C at night.

All micrographs were taken under 40x immersion oil objective.

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## Results and Discussion

The salt marsh microbial mat in Sippewissett MA contain a great variety of microorganisms stratified within the first 10 cm (top to bottom): gold layer (diatoms and cyanobacteria); upper green layer (cyanobacteria); pink layer (purple sulfur bacteria); peach layer (purple sulfur bacteria, mainly *Thiocapsa pfennigi*); lower green (green sulfur bacteria); and, black layer (sulfate reducing bacteria). The thickness and color intensity varies within short distances (10-20 cm), suggesting different stratification stages and/or local chemical composition. In the present study, few benthic invertebrates, such as nematodes, and some heterotrophic protists were observed. The initial sample was collected from a well developed site (all the layers present, see Figs. 1 and 2).

Six days after setting up the semisolid agar gradient tubes, different types of colonies appeared at different depths in the tubes at 1:100 dilution exposed to direct sunlight. Colonies were more abundant on the tubes prepared with 0.25% yeast extract than in the tubes prepared with boiled sterile mud. Three different types of colonies are clearly visible (see Fig. 3a): white and tiny colonies at the bottom of the tube, deep red colonies at the middle portion of the tube, and small light brown colonies close to the top sand overlay. The same type of spatial distribution of the colonies was observed on the tubes prepared with boiled sterile mud but they developed after 8-9 days (Fig. 3b).

The tubes buried in the wet sand bucket developed only two types of colonies after 6-7 days: white small colonies all over the tube, and black colonies close to and within the sand overlay (Fig. 4a, b), although some tubes presented black colonies all over the semisolid agar. Some white colonies turned blackish after awhile, and some tubes only developed black colonies. Tubes that were not completely buried in the sand developed red and brown colonies close to the top and in the sand plug (results not shown).

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Samples of each colony type are shown in figure 5 . There is no obvious cell shape difference within the white/black colonies developed in the buried tubes: motile rod-shape cells (Fig. 5a, b, c). The fact that the black colonies developed in buried tubes and at the bottom of these tubes, suggests that they could be sulfate reducing bacteria, although no gas analysis of the headspace was done. The brown upper layer developed in the tubes exposed to sunlight contain non-refractile round cells and few spirilla, all motile (Fig. 5d). The deep red middle colonies contain ribbon-shaped cells actively swimming (Fig. 5e, g). These two colony pigmentations suggest the presence of purple non sulfur bacteria. The white colonies developed at the bottom of the tubes contain rods, round and drumstick-shaped cells, not very motile (Fig. 5f, h). It should be noted that no refractile sulfur granules inside the bacteria were obvious thirteen days after inoculation, suggesting that sulfur bacteria were not enriched at this point in time, although they were present in the initial sample (see Fig. 2).

Colony stratification was not observed in tubes inoculated with higher inoculum concentrations (dilutions 1:10 and 1:50) and exposed to sunlight. Anoxygenic photosynthetic bacteria (red and brown colonies) spread all over the tube. However, seven days after inoculation, light green filamentous colonies developed close to the top (within the first 1-1.5 cm, see Fig. 6a). These green colonies are rich in diatoms and non-photosynthetic flagellates, as well as the enriched photosynthetic bacteria (see Fig 6b, c, d). At lower concentrations of inoculum, only bacteria were enriched at the same time point, suggesting that if any flagellate or other organisms with more or less the same nutritional requirements were present, they are not able to persist until the bacterial population reaches an optimal concentration for its survival.

Due to the experimental design, direct measurements of oxygen concentrations cannot be assessed. The sand overlay is a mechanical barrier that could damage the

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oxygen microsensors. However, a more qualitative approach was taken and tubes containing 25 mg/ml of rezazurin (oxygen colorimetric indicator) in the semisolid-inoculum (1:100 dilution) mixture were poured. Rezazurin tubes with and without inoculum were pink during the first 24 hours after pouring them; a day later, all the tubes turned clear, suggesting that an anoxigenic or at least microaerophilic environment was developed. It is very likely that a pH gradient is formed during the experiment, but the same constraints as with oxygen measurements apply in this case. A solution for pH measurements could be the presence of a pH universal indicator (wide pH range) in the semisolid agar, although toxicity and color interference with the bacterial population should be kept in mind. Another alternative is retrieving the intact semisolid agar/carbon source gradient, slice it and resuspend it in distilled water for pH electrode measurements.

It should be stressed out that the main objective of the present work was to test the possibility of stratified enrichment of a microbial mat community. No further isolations of organisms of each layer were pursued, but it would be worth trying them, in order to have a more precise description of the type of organisms enriched in this study. Also it should be considered that the development of the layers was followed only for three weeks. More organisms could appear beyond this time span. This observation suggests that an artificial species succession is taking place in the experimental model tested in this study, so it could be possible to isolate organisms not very abundant in the initial inoculum at the time the sample was taken.

### **Conclusions and Perspectives**

A gel stabilized system with an artificial carbon source can be used to obtain a partial stratification of some members of a salt marsh microbial mat community. This could be considered as an initial step of enrichment for several of these organisms. The present

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experimental design avoided chemical defined culture media; instead, rich media containing yeast extract or salt marsh mud were suitable to support the enrichment of some organisms. Many other residents could have been left out of this experimental set up, but further improvements can be made. Some suggestions follow: changing carbon source composition, include some inorganic chemicals, increase the surface area by including glass beads or thoroughly washed sand grains. It would be worth trying to test the possibility of studying succession under the experimental conditions tested in this work.

#### **Acknowledgments.**

I want to thank Dr. Thomas Pitta for the micrographs, for taking me to Sippewissett to collect the samples, and for encouraging me to continue this work; and, to The Department of Navy, Office of Naval Research for giving me financial support.

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## Figure legends

Fig. 1 Sliced microbial mat from the Salt Marsh, Sippewissett MA.

The most prominent layers are (from top to bottom): upper green (cyanobacteria), pink layer (purple sulfur bacteria), peach layer (purple sulfur bacteria, mainly *Thiocapsa*), and black layer (sulfate reducers). From the top layer to the beginning of the black layer there are 1.5-2.0 cm.

This picture was taken on July 22, 1995.

Fig. 2 Some residents of the Salt Marsh microbial mat.

Phase contrast micrographs from the resuspended mat used as inoculum. Cyanobacteria (a, c, d, h, where c is an epifluorescent micrograph of the bacteria shown in d). Sulfur bacteria (b, e, f), note a small spirochete in upper right corner in Fig. e, as well as the refractile sulfur granules inside the cells. A diatom (g).

Fig. 3 Partial stratification of some members of the microbial mat community in gel stabilized tubes exposed to direct sunlight.

Fig. a, tubes grown in 0.25% yeast extract; b, tubes grown in filtrated and boiled salt marsh mud. Note the interphase separation between the carbon source/agar at the bottom, and the white colonies developed in this zone. Also note the brown colonies close to the top sand overlay.

Fig. 4 Enrichment of sulfate reducer-like bacteria from the microbial mat in buried gel stabilized tubes.

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Note the development of a thick black zone within the sand overlay and tiny white colonies at the interphase of the semisolid agar/carbon source (a, yeast extract; b, boiled and filtrated salt marsh mud).

Fig. 5 Phase contrast of the stratified colonies developed under direct sunlight or in buried tubes.

Black layer observed in buried tubes and black colonies, a, b, c. Brown upper layer developed in the tubes exposed to sunlight, d. Deep red middle colonies, e, g. White colonies developed at the bottom of the tubes, f, h.

Fig. 6 High inoculum concentration (1:10) gel stabilized enrichments in tubes exposed to sunlight.

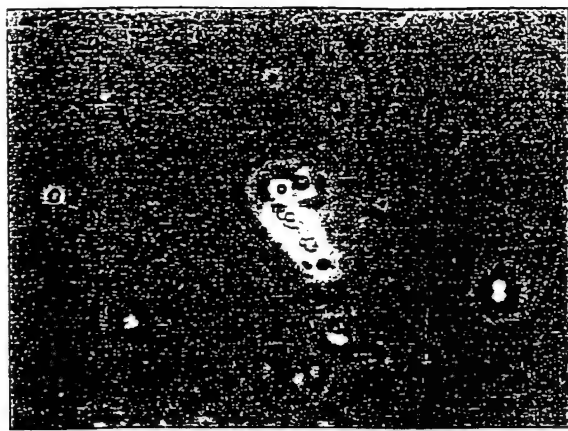
Gradient tube containing 0.25% yeast extract poured as slant, a. The tube was incubated for 7 days. Flagellated protists, b. Diatoms under phase contrast (c) or epifluorescence microscopy (d).



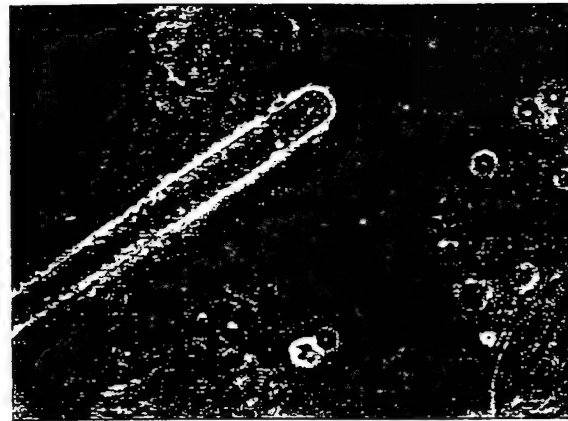
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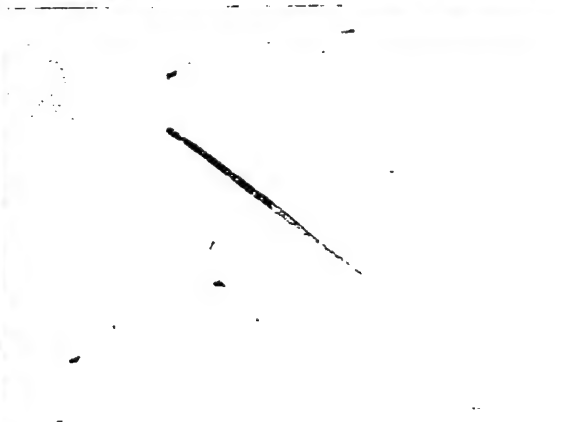
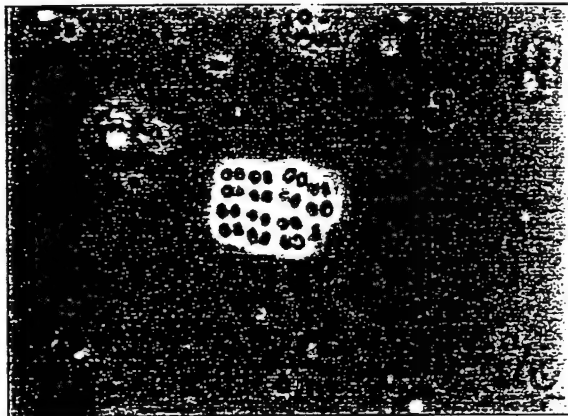
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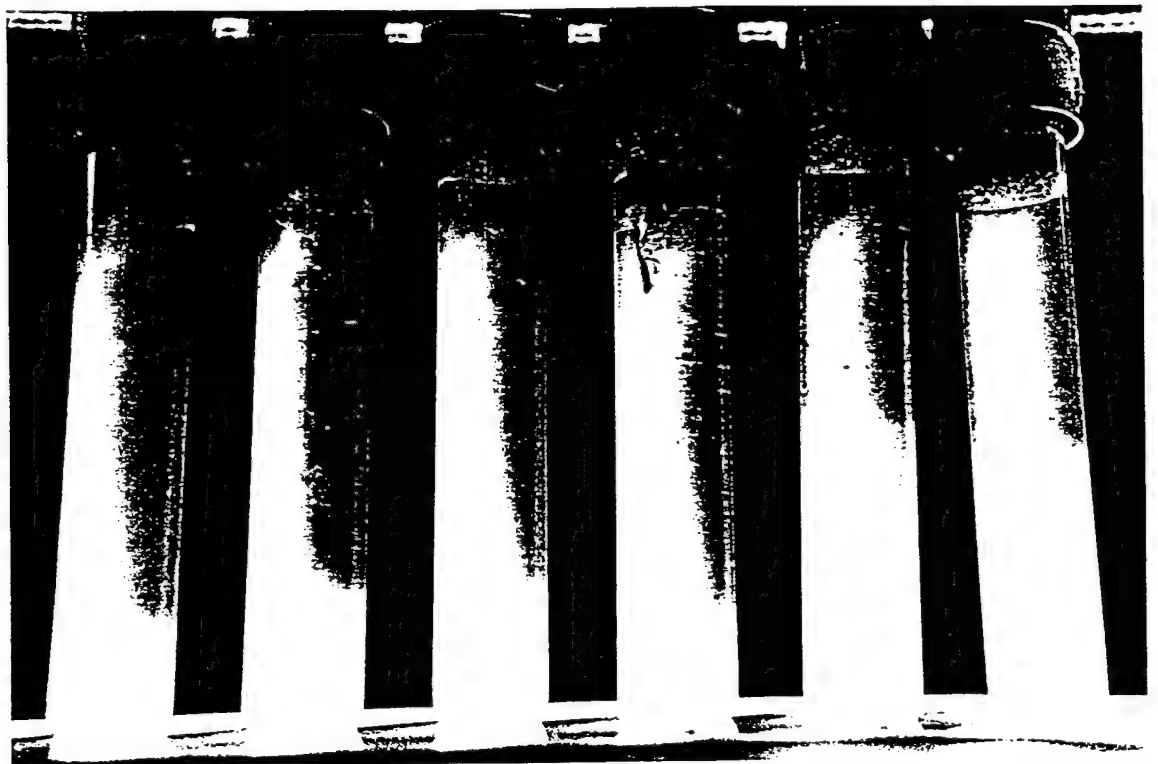
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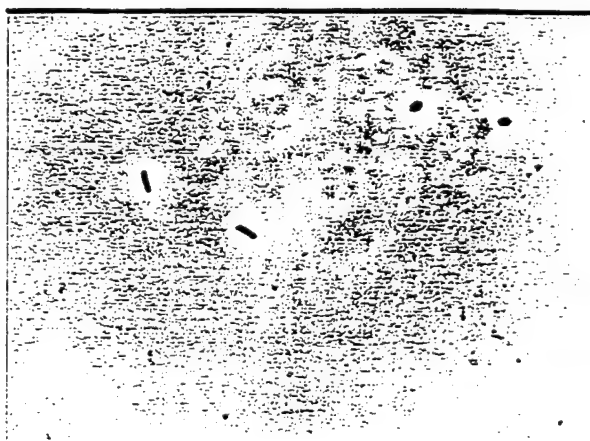
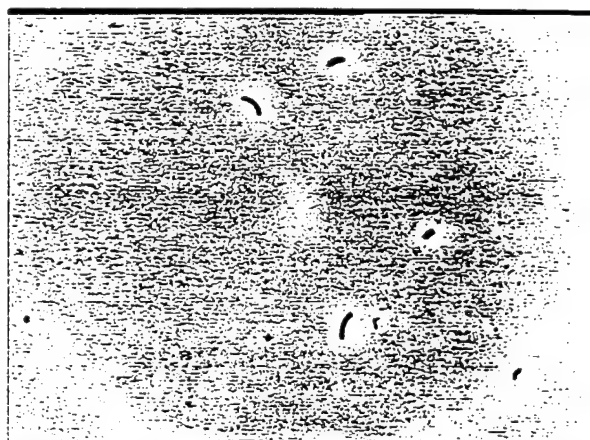
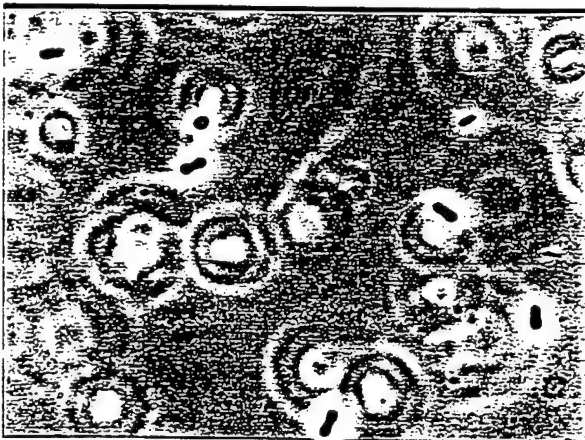
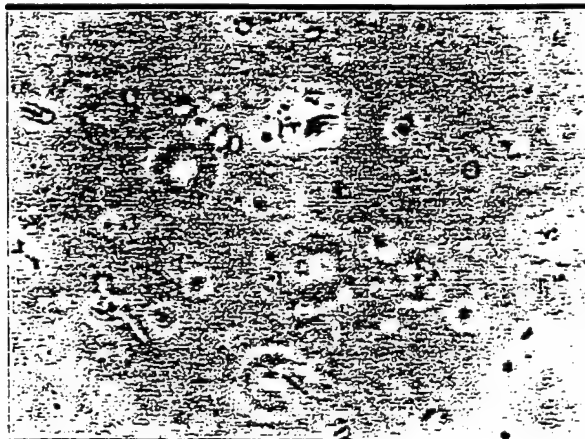
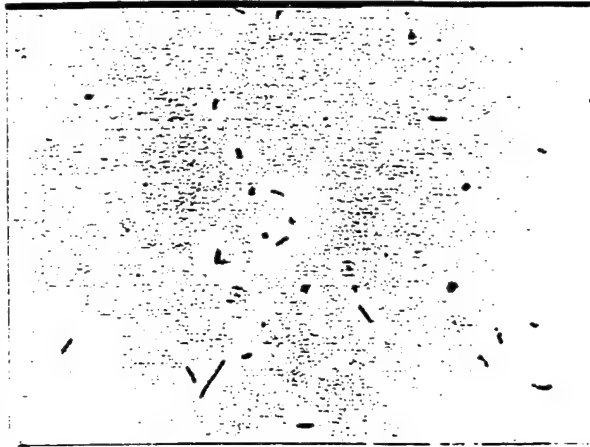
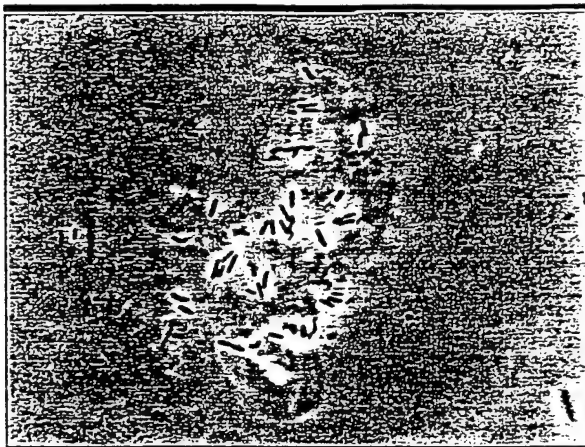
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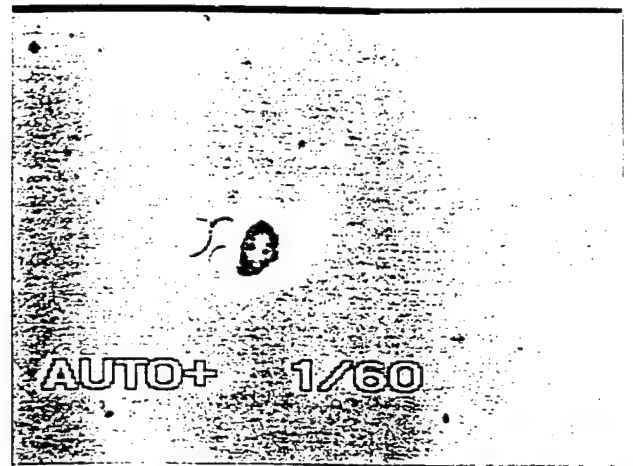
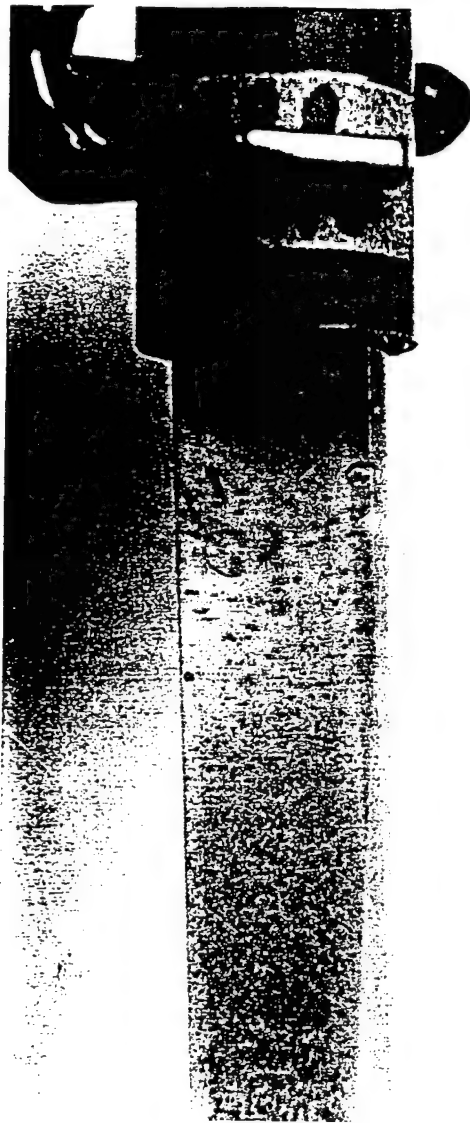


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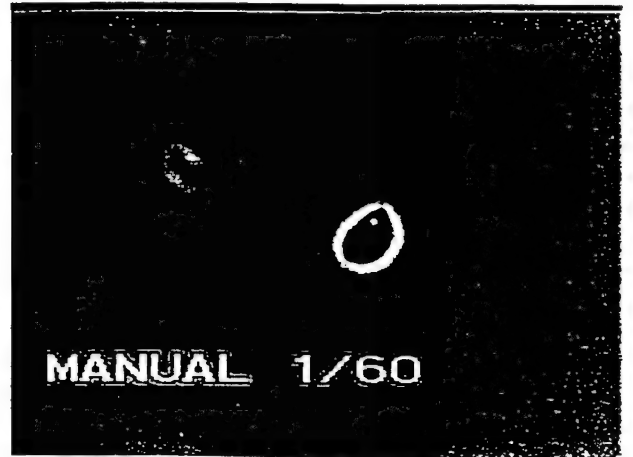
Fig. 1



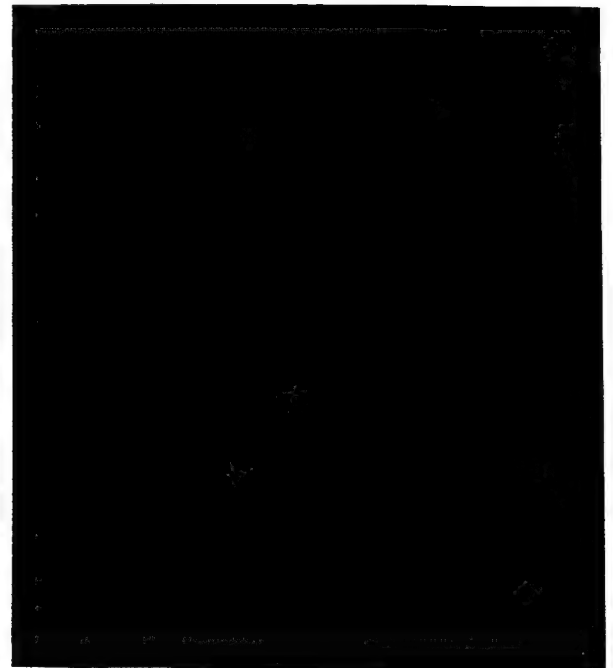
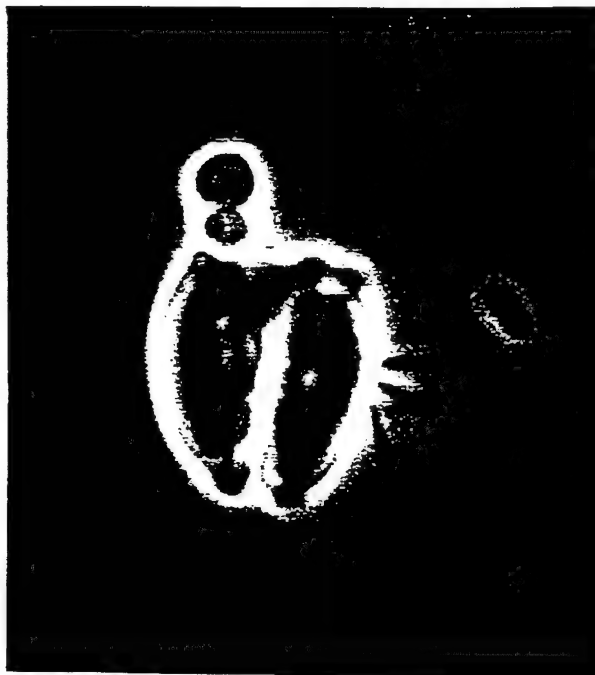
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MANUAL 1/60



**SIDE PROJECT**

**Bacteriophage extraction from the microbial mat from Sippewissett MA.**

**Elena Hilario\***

**Microbial Diversity Course**

**Summer 1995**

**Course directors: Dr. Abigail Salyers**

**Dr. Edward Leadbetter**

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## Bacteriophage extraction from the microbial mat from Sippewissett MA.

Elena Hilario

The aim of the present report was to obtain a crude extract of bacteriophages present in the microbial mat. The sample was not sorted out according to infected bacteria or free bacteriophages in order to obtain a great variety of viruses from the mat. The bacteriophage DNA was obtained as well as transmission electron micrographs of the intact viruses.

A sublibrary containing large bacteriophage DNA fragments digested with a six cutter restriction endonuclease could be cloned in a high copy plasmid able to produce single stranded DNA for sequencing. Positive clones can be sorted out according to their restriction pattern. Several groups are expected due to the heterogenicity of the DNA sample. Clones containing viral-related genes can be used to prepare non-radioactive probes (e.g. dioxygenin dUTP labeled). These probes could be used to screen bacterial isolates from the salt marsh in order to find out if they contain related viral genes and hopefully, find the host-virus system each probe belongs to.

The present approach could be very helpful for future studies on the role of viral control of bacterial population in the Salt Marsh Microbial Mat, as well as to study the genetic exchange among the residents of this community. This is a long term proyect that could be combined with the bacterial isolation projects done by the students of the Microbial Diversity Course, Woods Hole MA, interested in this topic.

Some useful readings are listed below:

Bratbak G, Thingstad F, Heldal M (1994) Viruses and the Microbial Loop. *Microb. Ecol.* 28, 209-221.

Proctor LM, Fuhrman JA (1990) Viral mortality of marine bacteria and cyanobacteria. *Nature* 343, 60-62.

Suttle CA (1994) The significance of viruses to martality in aquatic microbial communities. *Microbial Ecol.* 28, 237-243.

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## Procedure

- 1) A sample of ~50 ml of microbial mat from Sippewissett MA was collected in sterile Falcon tubes on July, 1995. The sample was kept at 4°C and processed two days later.
- 2) The sample was split in two Falcon tubes and resuspended with 100 ml of sterile TE buffer pH 8.0. The buffer was added stepwise, i.e 20-30 ml per tube each time until 100 ml of resuspended microbial population was obtained. The sample was vigorously shaken for about 10 min and let it stand for 15 min on ice.
- 3) Heat shock the resuspended sample 10 min at 70°C and place it on ice for 1 h.
- 4) Spin down at 6 800 x g, 4°C for 20 min. Transfer the clear supernatant into a sterile 250 ml beaker with magnetic bar without disturbing the bacterial pellet.
- 5) Add 5 grams of NaCl (final concentration 5 % w/v) to the supernatant (~90 ml recovered), stepwise, and stir slowly on a ice-water bath. Let it stand on ice for one hour.
- 6) Spin down the sample at 11 000 x g, 10 min, 4°C and save the supernatant in a sterile beaker with magnetic bar.
- 7) Add 9 grams of polyethyleneglycol (PEG 8000, final concentration 10%) stepwise to the sample and stir at room temperature for 30 min once all the PEG is dissolved (about 10 min). Chill the sample on ice for 1 h.
- 8) Spin down the sample at 11 000 x g, 10 min, 4°C. Discard the supernatant and resuspend the brownish pellet in SM buffer, final volume 4.5 ml. This is the crude bacteriophage extract.

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9) Spin down the phage extract in 3 microfuge tubes for 10 min at room temperature. A visible brownish pellet is obtained, but the supernatant is still brownish. Keep both samples at 4°C.

10) Resuspend one of the microfuge pellets to a final volume of 500 µl in TE buffer and extract it with equal volumes of phenol, phenol-chloroform-isoamyl alcohol, and chloroform. For each organic solvent extraction, the sample should be vigorously vortexed for 1 min and spin down at 10 000 rpm in a microfuge at room temperature for 2-3 min until an interface is observed.

11) Precipitate the final aqueous phase with 1/10 vol of sodium acetate 3 M pH 7.0, two volumes of cold ethanol, and store it at -20°C for 18 h, approximately.

12) Spin down the sample for 10 min at highest speed in the microfuge, at room temperature. Wash the pellet with cold 70% ethanol, spin down again and let the pellet dry at 37°C.

13) Resuspend the final pellet in 10 µl of TE buffer pH 8.0, add 2 µl of 6x loading buffer and run all the sample on a 1% agarose/ethidium bromide/1x TBE buffer gel.

#### Solutions:

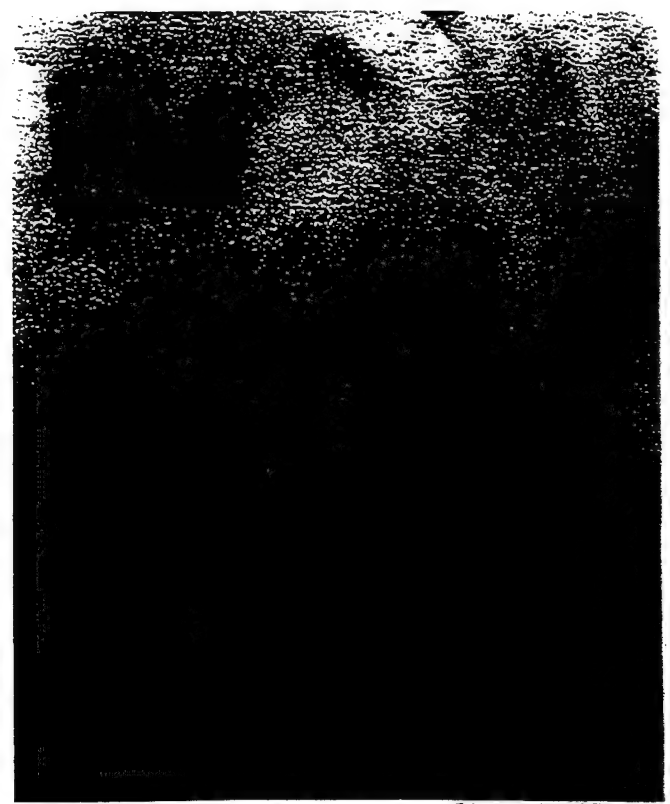
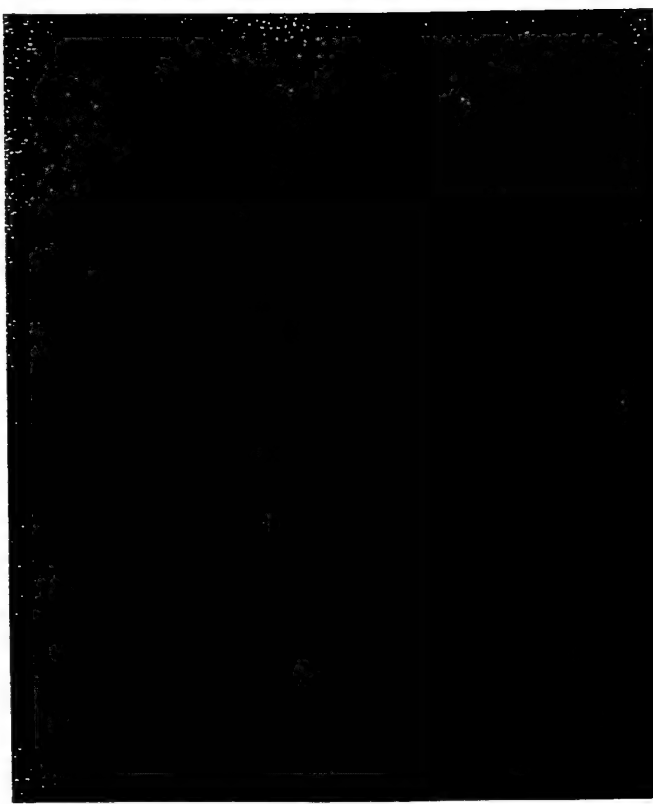
TE buffer     Tris-HCl 10 mM, EDTA 1 mM pH 8.0

SM buffer     NaCl 0.58%, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.2%, Tris-HCl 50 mM pH 7.5, gelatin 0.01%

#### Acknowledgments

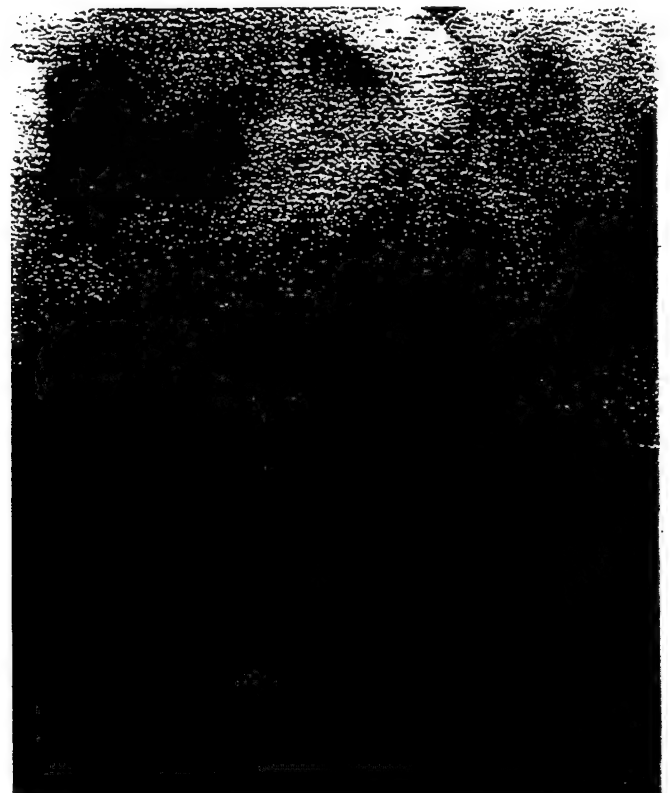
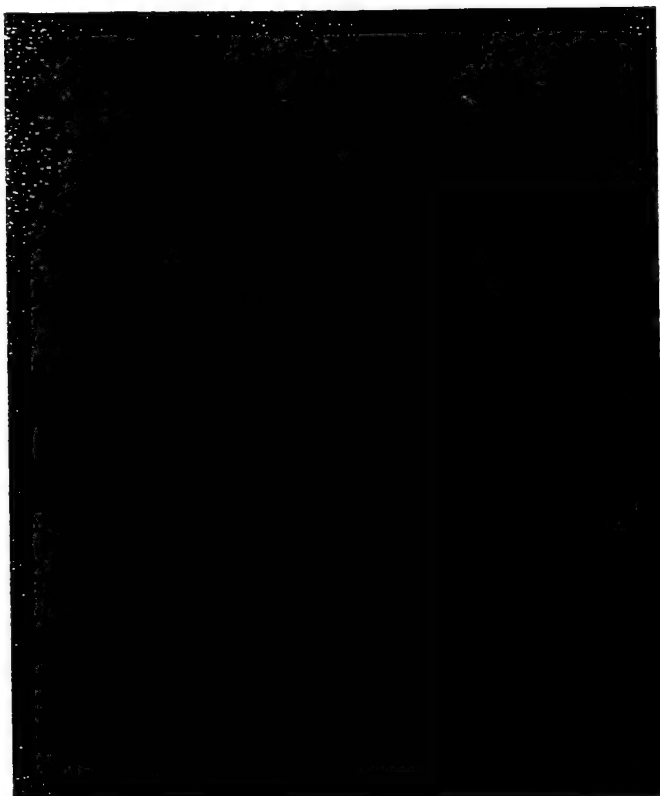
I want to thank Elizabeth Sherwood and Dr. Thomas Pitta for the transmission electron micrographs, Drs. Abigail Salyers and Edward Leadbetter for discussion and helpful hints.

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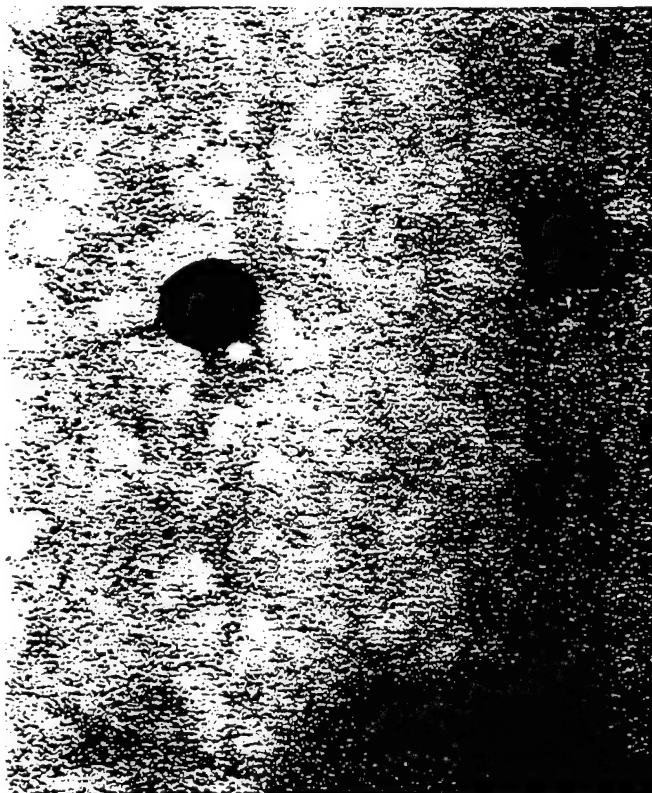


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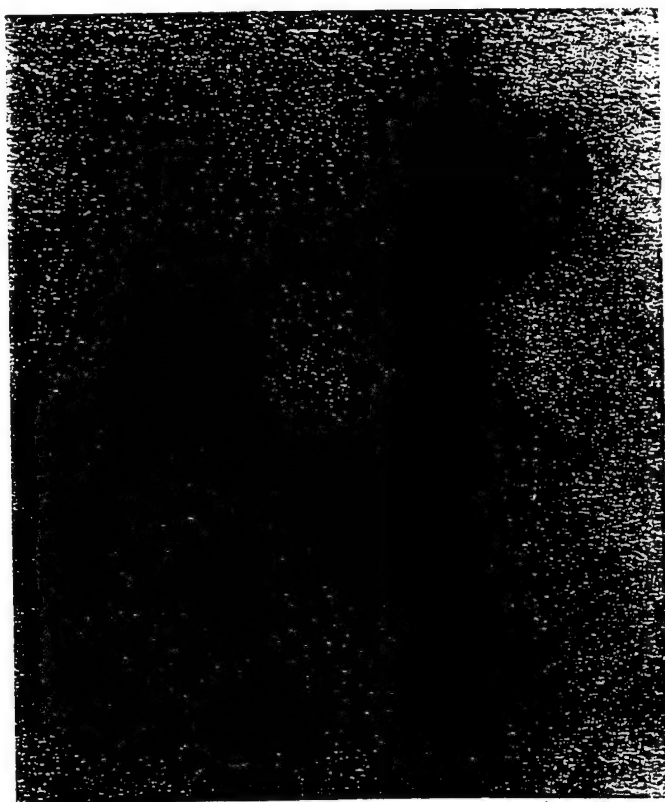
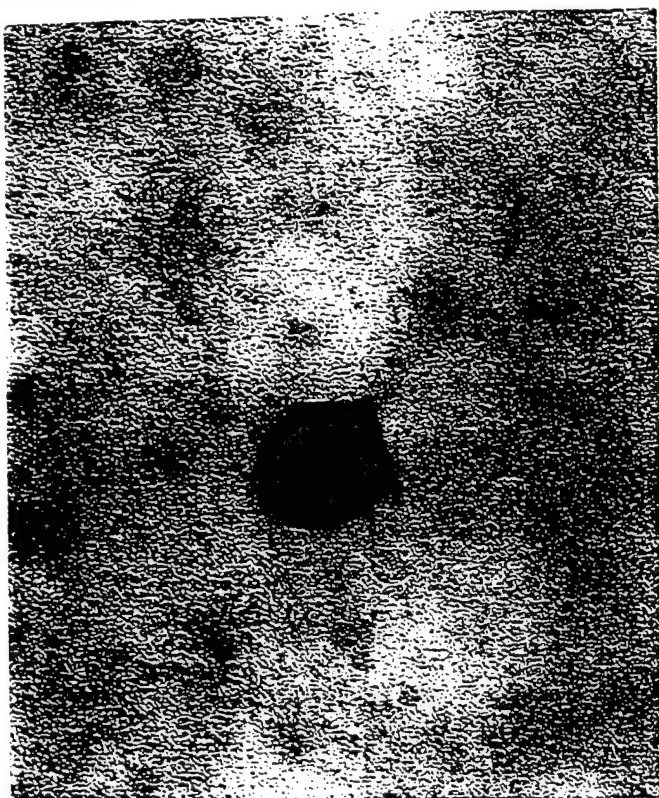
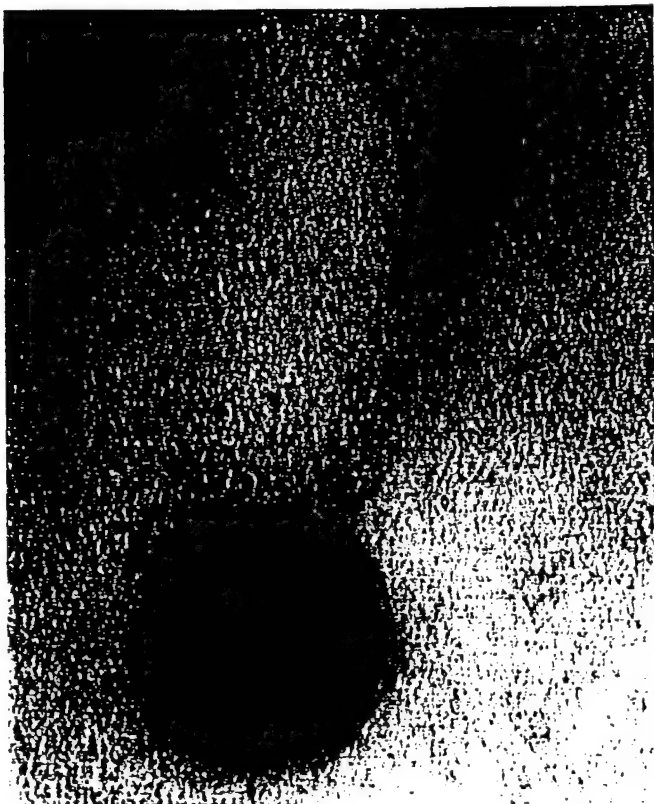




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## On the Use of Molecular Techniques for the Characterization of Methanogenic and Sulfate-Reducing Enrichments

Christof Holliger, EAWAG, Limnological Research Center, Kastanienbaum, Switzerland

### Abstract

Sediment from Cedar swamp was diluted with anaerobic mineral medium and incubated at 15°C and 35°C. Cellobiose was added to some of the cultures and methane formation and organic acid production was followed in time. In cultures incubated at 35°C where cellobiose was added, the pH dropped and a lot of organic acids such as propionate and butyrate accumulated. This process was slower at 15°C and in addition also acetate could be observed. Methane was formed in all cultures but only in small amounts. DNA was extracted from these cultures after 8 days of incubation. Even after purification and dilution of this DNA, it was not possible to obtain a PCR product of the 16S rRNA. It was therefore also not possible to study a possible shift in the methanogenic population by making bacterial community fingerprinting of amplified 16S rRNA gene sequences and restriction endonuclease analysis. The sediment sample from Buzzards Bay that was used to enumerate the sulfate-reducing bacteria contained  $2 \cdot 10^4$  SRBs per g dry sediment as estimated by a MPN count. It was impossible to enumerate the sulfate-reducing bacteria by *in situ* hybridization. Only a small percentage of the bacteria showed up after *in situ* hybridization with a universal probe compared with the amount of DAPI stained cells. The bacteria did apparently not contain enough ribosomal RNA. Although about 20-30% of the cells showed a signal with the universal probe, none could be detected with the SRB probe.

### Introduction

The use of gene probes for groups of bacteria makes it possible to observe specific bacteria directly in environmental samples. The probes are mainly designed with 16S rRNA as a target and there exist already a fair amount of specific probes and the number is increasing with every month. This new methodology perfectly complements the methods used so far in microbial ecology. Groups of bacteria were normally either

enumerated by plate counts or most probable number (MPN) counts. The activity was assessed by measuring metabolic products or by measuring the turn-over of labelled substrates. All methods on its own have their drawbacks. Either one found metabolic products but did not know what kind of bacteria were responsible for the formation or one found a group of bacteria present in an environmental sample but did not know whether it was metabolically active or not. With the gene probes technique one can visualize bacteria specifically in an environmental sample and quantify them. However, one can only visualize them if they are metabolically active since only cells which contain high enough amounts of ribosomal RNA can be detected. A combination of more traditional methods with molecular probing techniques makes it certainly possible to obtain a better insight in the microbial world in the environment.

The aim of this project is to apply molecular techniques to observe shifts in bacterial populations or to enumerate them. In methanogenic enrichments at mesophilic temperatures, methane formation is a result of hydrogenotrophic and acetoclastic methanogenesis, whereas at psychrophilic temperatures acetoclastic methanogenesis is the major methane forming process. The latter seems to be the result of a preferential hydrogen consumption by acetogens at low temperatures. The objective of this part of the project is therefore to investigate whether a difference of the methanogenic community can be observed if enrichments are incubated at 15°C and 35°C. The differences in the population will be evaluated by making bacterial community fingerprinting of amplified 16S rRNA gene sequences and restriction endonuclease analysis. In the second part of the project, the sulfate-reducing populations of a sediment sample of Buzzards Bay will be enumerated by either the MPN-method or by in situ hybridization and microscopically counting. The results of the two enumeration methods will then be compared.

## **Materials and Methods**

### *Source of inoculum*

For the methanogenic enrichments, sediment was collected from the Volta flame pond. It was stored at 15°C for four days before it was used. Buzzards Bay sediment for the experiment with sulfate reducers was collected during the cruise on July 1 and 2, 1995. It was stored at 15°C under aerobic conditions for 9 days prior to use.

### *Methanogenic enrichments*

The medium utilized contained (g/l, if not otherwise indicated):  $\text{KH}_2\text{PO}_4$ , 0.2;  $\text{NH}_4\text{Cl}$ , 0.25;  $\text{KCl}$ , 0.5;  $\text{CaCl}_2 \times 2\text{H}_2\text{O}$ , 0.15;  $\text{NaCl}$ , 1.0;  $\text{MgCl}_2 \times 6\text{H}_2\text{O}$ , 0.62;  $\text{NaHCO}_3$ , 2.5;  $\text{Na}_2\text{S}$ , 0.24; trace element solution SL-10, 1 ml; vitamin solution, 1 ml; vitamin  $\text{B}_{12}$ , 1 ml;

resazurin solution, 1 ml. The first six salts together with the resazurin solution were dissolved in 950 ml of water. The solution was heated until boiling and cooled under flushing with  $N_2/CO_2$  (4/1; v/v). An aliquot of 38 ml was dispensed in 150 ml serum bottles. The bottles were sealed with butyl rubber stoppers and the gas phase was changed with  $N_2/CO_2$  (4/1; v/v). After autoclaving, 1 ml of a filter-sterilized stock solution containing the vitamins and the trace elements and 1.2 ml of a stock solution containing  $NaHCO_3$  and  $Na_2S$  was added aseptically by syringe. To four bottles cellobiose was added from a stock solution to a final concentration of 25 mM. The enrichments were prepared in the anaerobic glovebox. Approximately 200 ml of the Volta flame pond sediment that contained a lot of decomposing leaves was washed with 100 ml medium and sieved through a mosquito net with mesh size of approximately 1 mm. This resulted in 180 ml sediment slurry. An aliquot of 18 ml was added to nine bottles with 40 ml medium. Outside the glovebox the gas phase was changed to  $N_2/CO_2$  (4/1; v/v) again. Two bottles without substrate and two bottles with substrate (cellubiose) were incubated at 15°C and 35°C, respectively. Methane was measured after 4 h of incubation and once every second day. A sample for the acetate measurement at time 0 was taken from the ninth bottle. From this bottle also three samples of 1.5 ml were collected and microcentrifuged for 5 minutes. The pellet was frozen until DNA extraction.

#### *MPN of sulfate reducers*

The medium utilized contained (g/l, if not otherwise indicated): Citric acid, 0.12;  $Na_2SO_4$ , 4.0;  $FeSO_4 \cdot 7H_2O$ , 0.1;  $KH_2PO_4$ , 0.2;  $NH_4Cl$ , 0.25;  $KCl$ , 0.5;  $CaCl_2 \cdot 2H_2O$ , 0.15;  $NaCl$ , 20.0;  $MgCl_2 \cdot 6H_2O$ , 3.0;  $NaHCO_3$ , 2.5;  $Na_2S$ , 0.02;  $Na_2S_2O_4$ , 0.02; trace element solution SL-10, 1 ml; vitamin solution, 1 ml; vitamin  $B_{12}$ , 1 ml; resazurin solution, 1 ml; Na-acetate, 0.8; Na-lactate, 1.1; Na-propionate, 0.6. The first nine salts together with the resazurin solution and the substrates acetate, lactate, and propionate were dissolved in 950 ml of water. The solution was heated until boiling and cooled under flushing with  $N_2/CO_2$  (4/1; v/v). An aliquot of 9.5 ml was dispensed in Hungate tubes. The tubes were sealed with butyl rubber stoppers and the gas phase was changed with  $N_2/CO_2$  (4/1; v/v). After autoclaving, 0.25 ml of a filter-sterilized stock solution containing the vitamins and the trace elements and 0.3 ml of a stock solution containing  $NaHCO_3$ ,  $Na_2S_2O_4$ , and  $Na_2S$  was added aseptically by syringe. The medium was still pink. One gram of sediment was added to one tube. Aliquots of 1 ml of this sediment slurry were transferred to three tubes by syringe. The sediment contained only very fine material and a 22 gauge did not get clogged. These tubes were considered to be the 100x dilutions of the MPN count. The dilutions were continued down to  $10^{-9}$ . The tubes were incubated at 30°C and FeS precipitation was checked to occur after 15 days of incubation.



#### *Extraction of cells followed by fixation versus direct fixation of cells*

Bacteria were extracted from Buzzards Bay sediment either by sonication or treatment with pyrophosphate±Nonidet P-40. For the extraction by sonication, 1 g of sediment was suspended in 10 ml PBS (130 mM NaCl, 7 mM Na<sub>2</sub>HPO<sub>4</sub>, 3 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.2), sonicated at low power three times for 10 s on ice. Subsequently, the slurry was vigorously vortexed for 1 min. The tubes were set on ice for 20 min, centrifuged in the table centrifuge for 2 min, and the supernatant was transferred to a new tube. The supernatant was centrifuged for 5 min at 5000 rpm in a centrifuge of the Physiology Course. The pellet was resuspended in 1.5 ml PBS and transferred to 2 ml tubes. The pellet was washed three times with PBS. Finally, the pellet was resuspended in 1.5 ml fixation buffer (40 g/l paraformaldehyde in 1xPBS, pH 7.2-7.4) and incubated at 4°C overnight.

For the extraction with pyrophosphate, 1 g of sediment was suspended in 10 ml of a 0.1% sodium pyrophosphate solution (pH 7.2). To one tube a drop of Nonidet P-40 was added in addition. These samples were subsequently treated as described for the sonication extraction after sonication.

For direct fixation, 1 g of sediment was suspended in 5 ml fixation buffer. After 2 min centrifugation in the table centrifuge, the supernatant was transferred to a new tube and the pellet was re-extracted with 2 ml fixation buffer. After subsequent centrifugation in the table centrifuge (2 min) the supernatants were combined and incubated at 4°C overnight.

The next morning, the samples were microcentrifuged, washed twice with PBS, and finally resuspended in 1 ml PBS/ethanol (1/1; v/v). They were stored at -20°C.

#### *DAPI staining*

Aliquots of 10 µl of the samples or of dilutions of it were applied on gelatine coated slides. After air-drying at 45°C, the sample was dehydrated in 50, 80, and 96% ethanol for 3 min and air-dried in a vertical position. On each well, 5 µl PBS and 5 µl DAPI solution (10 µg/ml) were applied. After 5 minutes incubation in the dark, the slides were rinsed with distilled water and air-dried. After dehydration and before DAPI staining, also *in situ* hybridizations were carried out.

#### *In situ hybridization*

The *in situ* hybridization was carried out as described in the general procedure (step 3 and further) of the handouts of Sandra Nierzwicki-Bauer with some modifications. The hybridization mix was warmed up to 45°C prior to application on the slides and the slides were rinsed with 1xSET after hybridization.

#### *DNA extraction, PCR amplification of ribosomal DNA fragments, and restriction with endonucleases*

The DNA was extracted from environmental samples after a protocol handed out during the course and developed by A. Sghir and J. Doré. The DNA was afterwards purified with the QIAEX II Gel Extraction Kit from QIAGEN or the XTreme Spin Column Kit from Pierce. The PCR was carried out following a protocol for Micro-PCR also provided by J. Doré.

#### *Analyses*

Methane was measured by GC/FID. Organic acids were analyzed by HPLC connected to a differential refractometer.

#### *Microscopy*

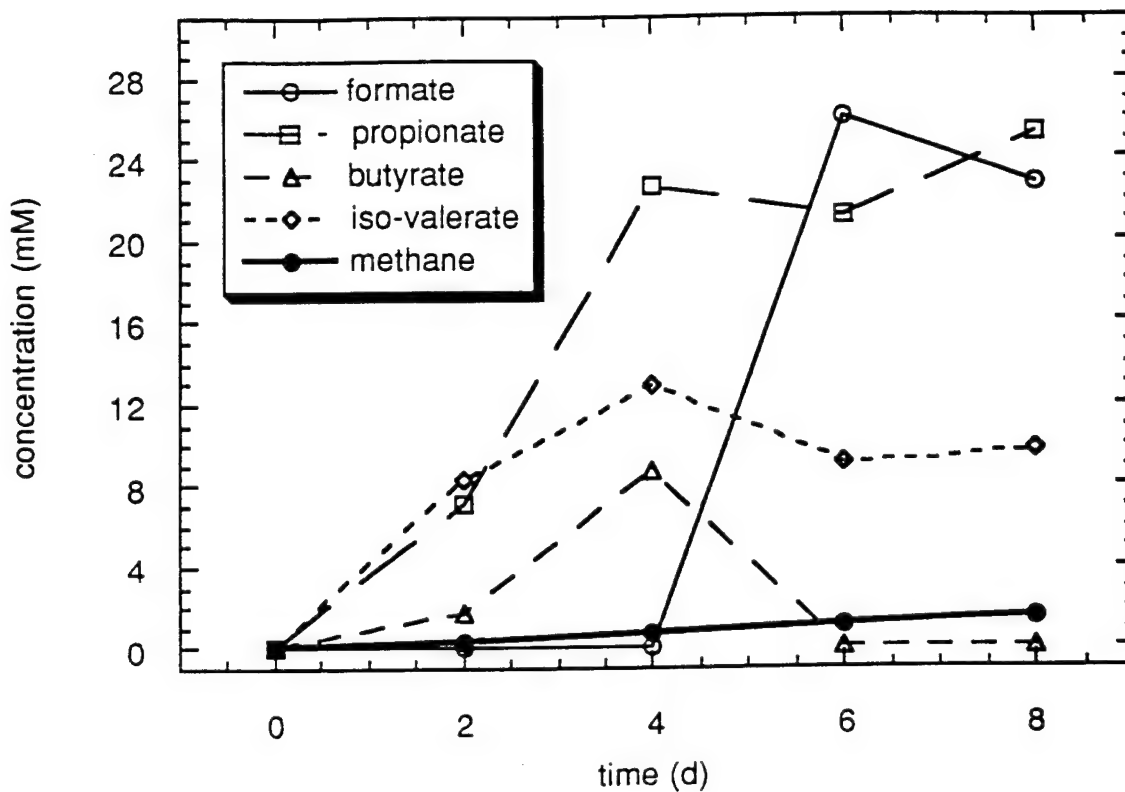
Slides with DAPI stained cells and cells after *in situ* hybridization were examined with a Zeiss Axioplan microscope equipped with the corresponding filter sets.

### **Results and Discussion**

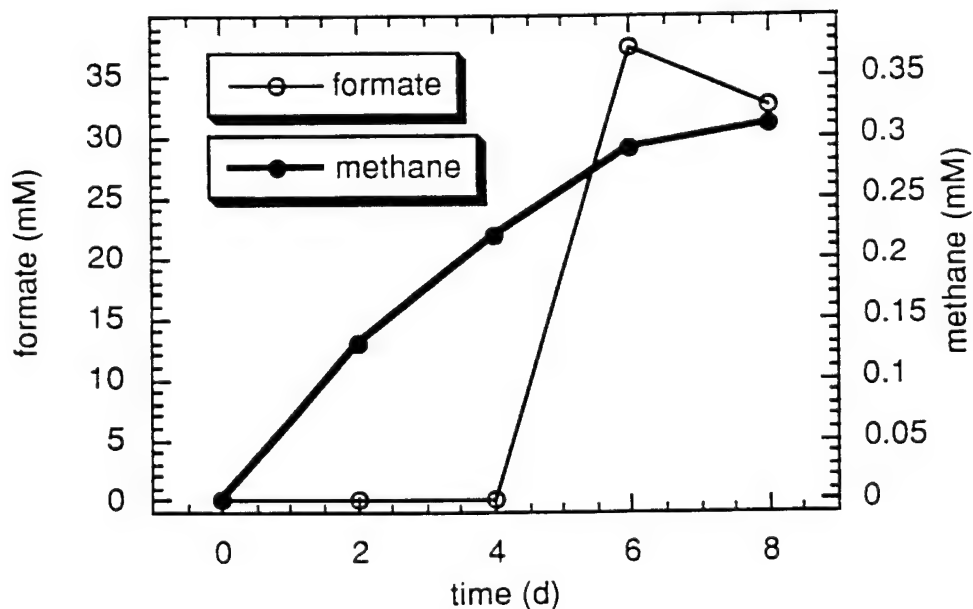
#### *Shift in the methanogenic population by making bacterial community fingerprinting of amplified 16S rRNA gene sequences and restriction endonuclease analysis*

Sediment from Cedar swamp was diluted with anaerobic mineral medium and incubated at 15°C and 35°C. Cellobiose was added to two cultures and two cultures did not obtain any additional substrate. Methane formation and organic acid production was followed in time. In cultures where cellobiose was added and which were incubated at 35°C, the pH dropped to pH 4.5 and a lot of organic acids such as propionate and butyrate accumulated (Fig.1). This process was slower at 15°C and in addition also acetate could be observed (Fig.3). The lack of acetate accumulation at 35°C is surprising since the low methanogenic activity in all cultures could not explain this phenomenon. The pH of the cultures was adjusted to neutral pH by adding sodium carbonate every second day. Methane was formed in all cultures but only in small amounts (Fig.1-4). The formate observed in all cultures at day 6 and 8 is probably a systematic error rather than actual formation in the cultures.

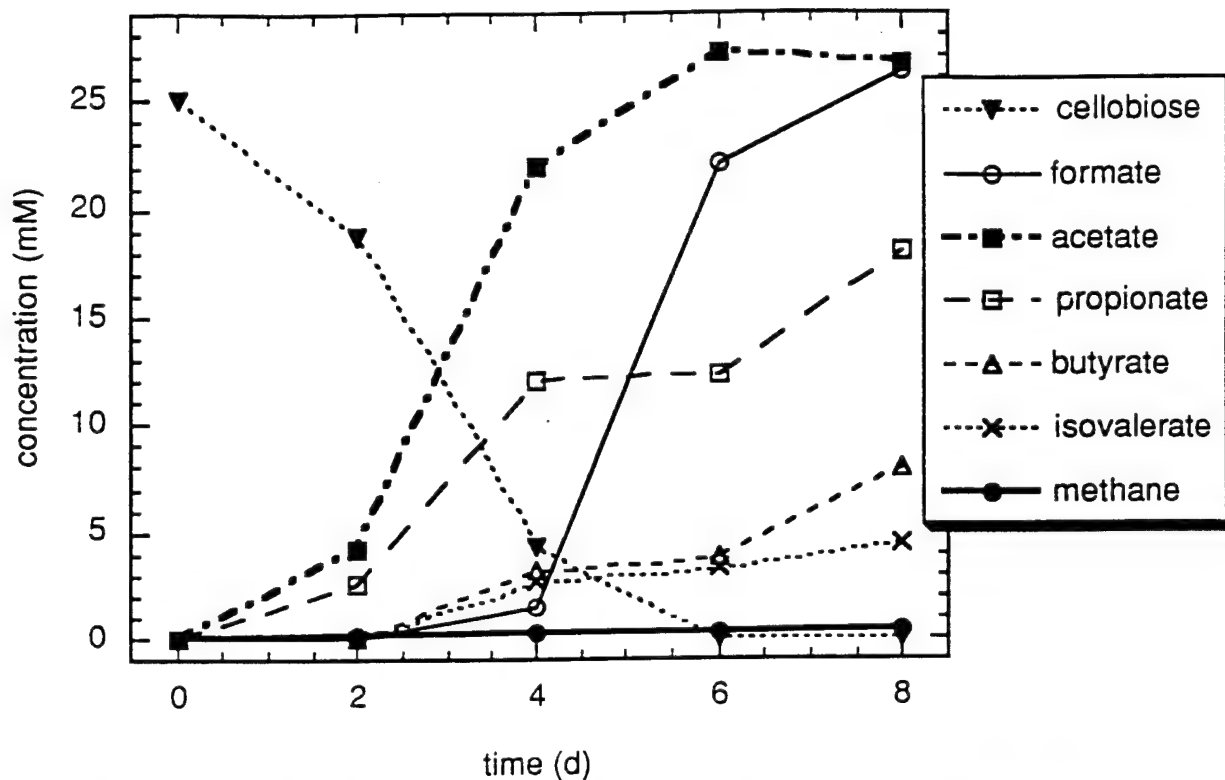




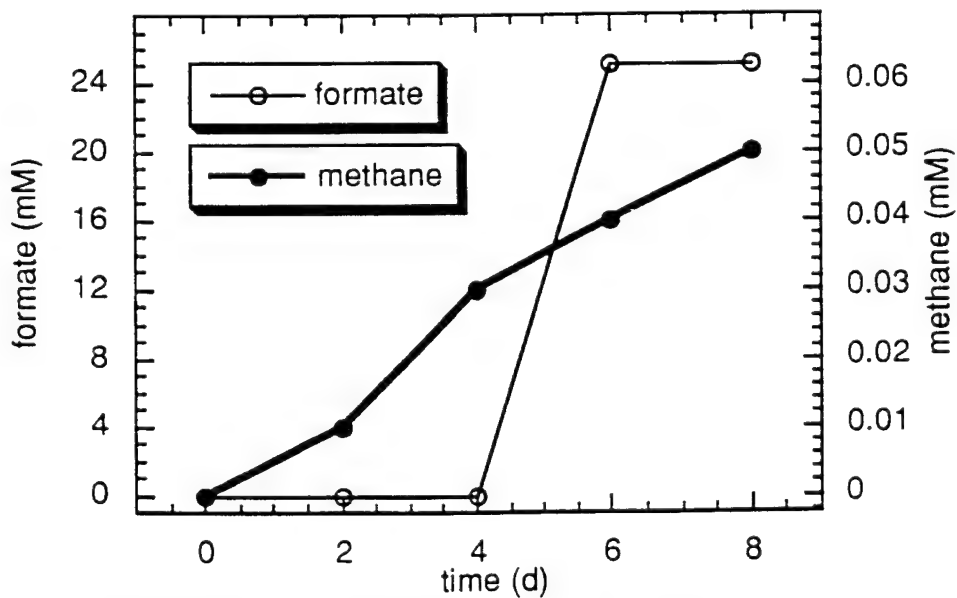
**Fig.1. Formation of methane and organic acids in a methanogenic enrichment at 35°C amended with cellobiose**



**Fig.2. Formation of methane and formate in a methanogenic enrichment at 35°C.**



**Fig.3. Formation of methane and organic acids in a methanogenic enrichment at 15°C amended with cellobiose**



**Fig.4. Formation of methane and formate in a methanogenic enrichment at 15°C.**

DNA was extracted from these cultures after 8 days of incubation. Even after purification and dilution of this DNA down to  $10^{-4}$ , it was not possible to obtain a PCR product of the 16S rRNA either with universal or archaea primers. The DNA was still brownish after the purification although much less compared with the color before the purification. The positive controls with DNA from *Bacteroides* and *Methanobrevibacter* showed that not one of reagents of the PCR kit was responsible for the lack of a PCR product but probably an inhibitory component still present in the DNA from the environmental sample, possibly humic substances. It was therefore not possible to test whether different fingerprints could be observed upon restriction of these PCR products with endonucleases and to obtain first indications of a shift in the methanogenic population after incubation at two different temperatures.

*Enumeration of the sulfate reducing bacteria in a sediment sample by the MPN method and by in situ hybridization*

The sediment sample from Buzzards Bay that was used to enumerate the sulfate-reducing bacteria either by the MPN method or *in situ* hybridization contained  $2 \cdot 10^4$  SRBs per g dry sediment as estimated by a MPN count. Tests with different extraction procedures to recover the cells from the sediment material compared with direct fixation of the cells showed that direct fixation was the most effective method. The sediment sample contained a lot of very fine particles that did not sedimentate very fast after extraction. Therefore the samples still contained high amounts of particles. For mounting the samples on the slides, they had to be diluted 100 times to obtained best resolution under the microscope. In addition, part of the material was washed off the slide when the 10 times dilution was applied. It was impossible to exactly enumerate the cells because they were not evenly distributed over the slide. DAPI stained cells were only observed in fields where also a lot particles were present. It was also impossible to enumerate the sulfate-reducing bacteria by *in situ* hybridization. Only a small percentage of the bacteria showed up after *in situ* hybridization with a universal probe compared with the amount of DAPI stained cells. Furthermore, the bacteria were only visible with the universal probe if the signal was integrated during 30 seconds. The bacteria did apparently not contain enough ribosomal RNA to allow detection after hybridization with 16S rRNA gene probes. Although about 20-30% of the cells showed a signal with the universal probe, none could be detected with the SRB probe. This is not too surprising provided that the amount of SRBs present in the sediment as determined by the MPN count was a good estimate of the exact amount of SRBs. The total amount of bacteria was not determined. However, if one would assume that approximately  $10^7$  bacteria per g dry sediment were present, the chance to see  $2 \cdot 10^4$  SRBs under the microscop is quite small.

## *Cultivation of Anaerobic Bacteria From the Human Vagina*

Dionne Hoskins, Microbial Diversity, Marine Biological Laboratory, Woods Hole Massachusetts, 02543

### **Introduction**

Very little is known about the ecology of anaerobic bacteria in the human vagina. Normally a lactobacillus- dominated environment, the vaginal tract usually has a pH of 4.5 (Rein, 1995). At this acidity there are few bacteria present which are not lactic acid producers. It is now known that the mature vagina is colonized by a variety of microbes, primarily obligate and facultative anaerobes. These include Lactobacillus acidophilus, Viridans streptococci, and Staphylococcus epidermis (Rein, 1995). Most population variation is attributed to hormonal fluctuations. Our descriptive knowledge of vaginal microbiology is primitive when it involves what other factors influence diversity.

Bacterial vaginosis (BV) is an infectious disease affecting the female reproductive system that is believed to be caused by a consortium of these anaerobic bacteria. Symptoms of BV include vaginal itching and excessive discharge; usually yellow-green in color and considerably malodorous. The offensive odor is thought to be caused by the production of cadaverine, putrescine, and trimethylamine. It is not known what triggers the change from a normal biota to the mixed, predominantly anaerobic community linked to vaginosis which includes Mobiluncus. No research to date has definitively described this heterogeneous mix of anaerobes.

The purpose of this research is to enrich for this poorly studied group of urogenital tract microorganisms. Because Mobiluncus is thought to co-occur with many other anaerobes, the approach of this study will be to optimize conditions for its growth. Other microbes which grow will be characterized along with the target organism. The investigation is aimed at culturing and characterizing Mobiluncus and other pathogenic anaerobes present in the vagina. Normal vaginal samples will be cultured using a variety of carbohydrate-enriched media. Cells will be identified through phase contrast microscopy, gram stain, catalase tests, and oxidase tests. Metabolic products will be detected using HPLC methodology.

### **Materials and Methods**

#### *Sample Preparation*

Vaginal smears were taken from two women (AS, DLH) complaining of no illness or symptoms. Smears were suspended in 5ml of composite media (described in media preparation). Two inoculating suspensions were made from each: one fresh and one cold treated. Fresh samples were simply resuspended and used for inoculation. Cold enriched samples were incubated at 20C for 45 minutes, then for 12 hours at 4C. This technique was employed because it has been demonstrated as encouraging the growth of Mobiluncus species (Smith & Moore, 1988).

#### *Media Preparation*

Four types of media were used in an effort to isolate as many different microorganisms as possible: 1% glycogen supplemented brain heart infusion agar (G), 1% glycogen supplemented brain heart infusion agar enriched with hemin (G+), .05% mucin supplemented brain heart infusion agar enriched with hemin (M), and sheep's blood brain heart infusion agar (B, available from BBL). Glycogen and mucin were provided as carbohydrate sources. Roll tubes were prepared using G, G+, and M agar for anaerobic cultures (N<sub>2</sub> w/ 10%CO<sub>2</sub> atmosphere). Enriched agars were prepared with hemin because many enteric microorganisms need an iron rich blood based medium. Sheep's blood BHI is a commercially available plate agar which has been successfully used for culturing Mobiluncus. (Smith and Moore, 1988; Hammann et. al., 1983). Cultures grown on brain heart infusion agars were later streaked onto blood plates and vice versa for comparative analysis.

A composite 1% glycogen media (.0005g hemin, 5g glycogen, .000375g vancomycin, .05 neomycin in 500ml distilled H<sub>2</sub>O) was prepared for use during sample collection and for liquid culture. Preexisting anaerobic basal media was often used for resuspension of cells when the composite media was too nutrient rich and would interfere with an assay.

### *Identification of Bacteria*

To elucidate characteristics of cultured cells, catalase, oxidase, gram stain, and hemolytic tests were performed. Gram stain and oxidase tests are commercially available (Difco). Hemolysis was assessed by observing isolates grown on sheep blood agar plates (BBL). Hemolytic organisms will show a golden (beta) or greenish (alpha) discoloration of blood agar. Hydrogen Peroxide (3%) was used for catalase assay.

For in situ hybridization, mature cells from 5 different plates and 3 used. Samples were suspended in anaerobic basal media and chilled on ice for 5 minutes. The sample (1 ml) was centrifuged at top speed for 5 minutes. The pellet was resuspended and washed twice in iced PBS, centrifuging after each wash. Afterwards, the final pellet was resuspended 1:100 in cold PBS and applied to subbed slides in 15 microliter aliquots. Subbed slides were well slides which had been washed 20 times in H<sub>2</sub>O, twice in dH<sub>2</sub>O, baked 4-8 hours at 200C, and then dipped in subbing solution (0.1% gelatin and 0.1% CrK(SO<sub>4</sub>) 12H<sub>2</sub>O). They were dried in a 37C incubator. Before adding the probes, culture slides were treated with ethanol/formaldehyde solution (90:10) for 5 minutes at room temperature. To make sure all fixative was removed, slides were rinsed twice in dH<sub>2</sub>O and air dried. Low G+C, high G+C, and flavobacteria probes (40microliters) were applied to each well and incubated overnight. Low G+C and flavobacteria hybridizations were incubated at 45C. High G+C samples were incubated at 37C. After incubation slides were washed three times in 1XSET solution at 37C and examined using oil immersion epifluorescent microscopy.

### **Results**

White, opaque colonies appeared in 24 hours on all plates. Plate colonies matured in approximately 48 hours, becoming more tacky with time. Young and mature colonies all had a distinctive smell of trimethylamine. The initial roll tube enrichments took about 3 days to grow and were much smaller, more wet colonies. However, all following reinoculates of tubes grew overnight.

Bacteria fell into the following morphological types: cocci (single cells, chains, clusters, diploids, tetrads), coccobacilli (single cells and diploids), and bacilli. Most cultures were dominated by cocci and coccobacilli (Table 1).

Cold enrichments were discontinued after the third streaking because the colonies were all identical to each other and not significantly different from the fresh samples. Similarly, the anaerobic plates were discarded because the growth on them was the same as that on the aerobic plates. As stated earlier, two women provided sample specimens, but after several streaking attempts the culture populations showed no difference among G+, M, and G plates. Subsequently, only DLH cultures were continued, the only exception being the blood agar plates.

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G+ plate	short bacilli
M plate	small cocci
G plate	small cocci
Blood plates	small diplococci, short coccobacilli, elongate bacilli, short diplococcobacilli
G+ roll tube	short coccobacilli
M roll tube	short coccobacilli
G roll tube	cocci

\*G+, 1% glycogen enriched w/hemin; M, .5% mucin enriched w/hemin; G, 1% glycogen; B, sheep's blood brain heart infusion

Only approximately 30% of the samples were catalase negative (Table 2). The oxidase test revealed almost an even division of oxidase negative and positive cells. The gram stain was positive for all samples tested.

Table 2. Catalase, Oxidase, and Hemolytic Test Results

Sample	Oxidase	Catalase	Hemolysis
1	+	+	B
2	-	+	B
3	-	+	B
4*	-	+	none
5*	-	+	B
6*	+	+	B
7	+	+	A
8*	+	+	none
9*	+	+	none
10*	+	+	none
11	+	+	none
12	-	-	none
13	-	-	none
14	-	-	none
15	-	-	none
16	-	-	none

B- beta hemolytic, A- alpha hemolytic

\*AS blood plate sample, all others are DLH

In situ hybridization conducted on all cultures used high G+C, low G+C, and flavobacteria specific probes. Of the 8 samples tested for each probe, 2 of the low G+C showed positive (Table 3).

## References

- Hammann, R., A. Kronibus, A. Viebahn, and H. Brandis. 1984. Falcivibrio grandis gen. nov. sp. nov., and Falcivibrio vaginalis gen. nov. sp. nov., a new genus and species to accommodate anaerobic motile curved rods formerly described as "Vibrio mulieris" (Prevot 1940). Breed et. al. 1948. Syst. Appl. Microbiol. 5:81-96.
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## Introduction

Very little is known about the ecology of anaerobic bacteria in the human vagina. Normally a lactobacillus- dominated environment, the vaginal tract usually has a pH of 4.5 (Rein, 1995). At this acidity there are few bacteria present which are not lactic acid producers. It is now known that the mature vagina is colonized by a variety of microbes, primarily obligate and facultative anaerobes. These include *Lactobacillus acidophilus*, *Viridans streptococci*, and *Staphylococcus epidermis* (Rein, 1995). Most population variation is attributed to hormonal fluctuations. Our descriptive knowledge of vaginal microbiology is primitive when it involves what other factors influence diversity.

Bacterial vaginosis (BV) is an infectious disease affecting the female reproductive system that is believed to be caused by a consortium of these anaerobic bacteria. Symptoms of BV include vaginal itching and excessive discharge; usually yellow-green in color and considerably malodorous. The offensive odor is thought to be caused by the production of cadaverine, putrescine, and trimethylamine. It is not known what triggers the change from a normal biota to the mixed, predominantly anaerobic community linked to vaginosis which includes *Mobiluncus*. No research to date has definitively described this heterogeneous mix of anaerobes.

The purpose of this research is to enrich for this poorly studied group of urogenital tract microorganisms. Because *Mobiluncus* is thought to co-occur with many other anaerobes, the approach if this study will be to optimize conditions for its growth. Other microbes which grow will be characterized along with the target organism. The investigation is aimed at culturing and characterizing *Mobiluncus* and other pathogenic anaerobes present in the vagina. Normal vaginal samples will be cultured using a variety of carbohydrate-enriched media. Cells will be identified through phase contrast microscopy, gram stain, catalase tests, and oxidase tests. Metabolic products will be detected using HPLC methodology.

## Materials and Methods

### *Sample Preparation*

Vaginal smears were taken from two women (AS, DLH) complaining of no illness or symptoms. Smears were suspended in 5ml of composite media (described in media preparation). Two inoculating suspensions were made from each: one fresh and one cold treated. Fresh samples were simply resuspended and used for inoculation. Cold enriched samples were incubated at 20C for 45 minutes, then for 12 hours at 4C. This technique was employed because it has been demonstrated as encouraging the growth of *Mobiluncus* species (Smith & Moore, 1988).

### *Media Preparation*

Four types of media were used in an effort to isolate as many different microorganisms as possible: 1% glycogen supplemented brain heart infusion agar (G), 1% glycogen supplemented brain heart infusion agar enriched with hemin (G+), .05% mucin supplemented brain heart infusion agar enriched with hemin (M), and sheep's blood brain heart infusion agar (B, available from BBL). Glycogen and mucin were provided as carbohydrate sources. Roll tubes were prepared using G, G+, and M agar for anaerobic cultures ( $N_2$  w/ 10%  $CO_2$  atmosphere). Enriched agars were prepared with hemin because many enteric microorganisms need an iron rich blood based medium. Sheep's blood BHI is a commercially available plate agar which has been successfully used for culturing *Mobiluncus*. (Smith and Moore, 1988; Hammann et. al., 1983). Cultures grown on brain heart infusion agars were later streaked onto blood plates and vice versa for comparative analysis.

A composite 1% glycogen media (.0005g hemin, 5g glycogen, .000375g vancomycin, .05 neomycin in 500ml distilled  $H_2O$ ) was prepared for use during sample collection and for liquid



culture. Preexisting anaerobic basal media was often used for resuspension of cells when the composite media was too nutrient rich and would interfere with an assay.

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Table 3 Results of In Situ Hybridization

Sample	Fluorescence	Morphology
A	-	large & small cocci
B	-	diplococci
C	+	chained & single diplococci
D	-	short & long bacilli
E	+	diplococcobacilli
F	-	bacilli, cocci
G	-	diplo & chained cocci, bacilli
H	-	short bacilli

## Discussion

It was expected that most cells would be gram positive rods or cocci and that trimethylamine would be produced. Although the gram staining kit used (Difco) was not very accurate, it was largely indicative that the cells, mixed cultures and all, were gram positive. Catalase and Oxidase results were expected to be variable and were. These tests, along with the morphotyping, were done primarily to narrow down what the cells may be in preparation for the in situ hybridization. It is imprecise to comment on the production of trimethylamine because the HPLC was not accessible. Equipment modifications were needed that did not fit into the time period allotted for the experiment. However, it will suffice to say a distinct, pungent odor suggestive of trimethylamine was produced by all cultures. General comparisons between samples and pure solutions of cadaverine and putrescine support this assumption.

The cold enrichment technique was ineffective since it yielded no rod bacteria, although it has been recommended for cultivating Mobiluncus species. The possible explanations for this are that

- 1) the sample provider(s) were not infected
- 2) the bacilli were killed during the procedure
- 3) other opportunistic microbes overcrowded the target organisms

Another interesting phenomenon was the similarity between the aerobic and anaerobic cultures. This is most likely explained by the inability of the anaerobic jars to remain anoxic. At best the atmosphere was microaerophilic and therefore was unsuitable for obligate anaerobes as Mobiluncus and other cells.

In situ hybridization and lab analyses reveal that the positive samples probably contain Staphylococcus, Streptococcus, and Lactobacillus. Gram stain, catalase, oxidase, and probe data eliminated the possibility of there being Bacteroides, Gardnerella, Trichomonads, Neisseria, or Chlamydia.

Because of the similarity of morphotypes and lack of extraction methods, it's difficult to positively identify species. Future efforts should focus on GC and HPLC methods for quantifying amines to better discern between the existing anaerobic biota.

## References

- Hammann, R., A. Kronibus, A. Viebahn, and H. Brandis. 1984. Falcivibrio grandis gen. nov. sp. nov., and Falcivibrio vaginalis gen. nov. sp. nov., a new genus and species to accommodate anaerobic motile curved rods formerly described as "Vibrio mulieris" (Prevot 1940). Breed et. al. 1948. Syst. Appl. Microbiol. 5:81-96.
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# Survey and Isolation of Bacteriophages of Photosynthetic Purple Non-Sulfur and Marine Luminous Bacteria

Eric S. Miller [Department of Microbiology, North Carolina State University, Raleigh, NC 27695]

Marine Biological Laboratory, Woods Hole, MA  
Microbial Diversity Course 1995

## Abstract

A survey of the bacteriophages in fresh and marine waters of the Woods Hole, Mass. area was conducted. Twelve environmental isolates of purple non-sulfur bacteria, that likely included species of *Rhodobacter*, *Rhodopseudomonas*, *Rhodomicrobium* and *Rhodospirillum*, were used as hosts to identify plaque-forming units. Although several water sources were examined (School Street Marsh, Cedar Swamp, Lilly Pad Pond, etc.) no plaque forming phage were identified. Lysogenic strains were screened but they also were not found. However, in these latter studies, three potential phototrophic bacteriocin-producers were noted. Transmission electron microscopy (TEM) of one concentrated fresh water sample revealed the presence of several phage. Further studies are needed to identify productive phage-host pairs from these habitats, and to enhance the yield of plaque-forming phage. Three environmental isolates of luminous *Vibrio* (two probable *V. fischeri* and one *V. harveyi*) and one laboratory strain of *V. fischeri*, were used as hosts to survey sea water. Using the methods employed for screening fresh water, phage again were not obtained. Subsequently, a co-culture amplification procedure was successfully employed to yield a plaque-forming vibriophage and a probable bacteriocinogenic *Vibrio*. Preliminary host range characterization of the newly isolated phage,  $\phi$ MBL-1, was conducted.

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## Introduction

Viruses of prokaryotes (e.g., bacteriophages) appear to occur in essentially all habitats occupied by the Bacteria or Archaea. Those environments for which the occurrence of bacteriophages has been surveyed include, but is not limited to, soils, marine and fresh waters, aerobic and anaerobic environs of multicellular Eukarya and the "extreme" environments frequently occupied by Archaea (e.g., hypersaline and thermophilic). The number of bacteriophages present in environmental samples appears to vary from very few to as many as  $10^5$  -  $10^6$  per ml of estuarine and pelagic marine waters. The vertebrate gastrointestinal tract contains numerous DNA and RNA phages per gram of feces.

Despite the prevalence of bacteriophages in ecologically significant environments, little is known, for example, of their influence on microbial population dynamics (e.g., "grazing"), intra- or inter-species gene transfer, or microbial evolution or adaptation. Although the occurrence, and often the morphology, of prokaryotic viruses from natural environments have been described, their molecular or genomic characterization is often lacking. The two-volume monograph *The Bacteriophages* (1) contains only two chapters focusing on non-coliphage viruses. In the several thousand page volume *The Prokaryotes* (2), a scant two dozen genera are discussed with respect to isolated phages, most of which are only used for strain "typing". Therefore, a thorough characterization of virus-bacterial relationships from natural habitats can provide insights to the metabolic and genomic activities therein, and molecular characterization of prokaryotic viruses from diverse habitats can potentially yield new enzymes or molecules of biotechnological interest.

The objectives of this short-term project were to survey the occurrence, and potentially isolate, bacteriophages that infect bacteria of fresh and marine water habitats. The purple non-sulfur photosynthetic bacteria (3, 4) and the luminous marine *Vibrios* (5) were used as the fresh water and marine bacterial hosts. The presence of temperate bacteriophage in bacteria (lysogens) newly retrieved from the two environments was evaluated. In parallel determinations, the presence of lytic phage in the respective waters was examined by transmission electron microscopy.

## Materials and Methods

**Media.** Photosynthetic purple non-sulfur bacteria were grown in liquid PNSB minimal medium (2); PNSB agar plating medium contained 1.5% 4X washed Difco agar. Complete medium (PYVS) consisted of 0.3% peptone, 0.5% soytone, and 0.3% yeast extract;

PYVS agar plating medium contained 1.5% agar. Soft top agar overlay media was as above, except the final agar concentration was 0.75 %. All reagents were from Difco.

Luminous bacteria were grown in sea water complete medium (SWC; 0.5% peptone, 0.5% tryptone, 0.5% yeast extract and 0.3 % glycerol. in 70% sea water). SWT medium contained 0.3% tryptone, 0.3% yeast extract and 0.3% glycerol in 70% sea water. Agar plating and soft top agar contained 1.5% and 0.75% Difco agar, respectively.

Mitomycin C (Sigma Chem., St. Louis, MO) was prepared as a sterile 0.2 mg/ ml dH<sub>2</sub>O solution stored at 10°C in the dark.

**Bacteria.** Photosynthetic purple non-sulfur bacteria were isolated by course participants from a fresh water marsh (School Street Marsh; Woods Hole, MA) by vacuum filtration of 1 to 10 ml of water sample onto 47 mm 0.4  $\mu$ m cellulose acetate disks. Disks were incubated on PNSB minimal agar medium in Gas-Pak H<sub>2</sub>/CO<sub>2</sub> jars, illuminated with a 90 W GE tungsten lamp (8 - 12 inch distance), until colonies developed (approximately 4 days). Individual colonies were streaked two or more times onto PNSB agar and incubated as above until apparently axenic. Liquid PNSB cultures were obtained by inoculating 18 ml screw-cap tubes with isolated phototrophs, and incubating the closed tubes in the light for 2 - 4 days in PNSB.

Luminous marine bacteria were isolated on SWC agar medium by spreading 0.05 - 0.2 ml of water from surf (MBL Beach) or harbor (Eel Pond) sources, or by spreading a one loop volume of oyster gill (Garbage Beach; via Marine Resources Center) or gut material directly onto the agar surface. All samples were obtained in Woods Hole, MA. After approximately 15 hr at room temperature, luminous colonies were identified, transferred to SWC agar and streaked until apparently axenic. *Vibrio fischeri* ( $\Delta luxI$ ) was obtained from P. Dunlap, WHOI.

**Bacteriophage plaque assays.** Lytic bacteriophage were surveyed by mixing 0.05 - 0.1 ml of fresh or sea water samples with 0.05 - 0.2 ml of bacterial culture. Water samples were variably treated by filtration through glass fiber, 0.4  $\mu$ m nylon polycarbonate, or concentration with 0.02  $\mu$ m Acrodisks. For phototrophs, 2 day, turbid PNSB cultures were used as host cells. For luminescent bacteria, freshly-grown overnight, or 2 - 6 hr cultures (room temperature in SWT broth) were used. Where indicated, CaCl<sub>2</sub> and MgSO<sub>4</sub> were each added to the plating mixtures at 1 mM. After 15 min at rm. temp., cell/ water mixtures were added to 2.5 ml of molten top agar (48C) and distributed over the surface of PNSB or PYVS (phototrophs) or SWT (*Vibrios*) agar plates. Plates were incubated at rm temp for each cell type (as described above) and the presence of plaques evaluated over a 1 - 4 day period.

**Prophage induction.** Temperate bacteriophage were screened by incubating phototrophic or luminous bacteria in liquid PNSB or SWC medium, respectively, that contained 0.2  $\mu$ g/ml Mitomycin C. Following 12 - 15 hr incubation (cell lysis was variably observed), one ml of the culture was clarified by microcentrifugation (twice) at full speed. Culture supernatants were used in plaque assays as described for lytic phage, or a one-loop volume was streaked onto the surface of an agar plate and then overlaid with top agar containing indicator cells. CaCl<sub>2</sub> and MgSO<sub>4</sub> were variably added to 1 mM.



**Amplification of phage in sea water.** 250 ml of sea water (collected from Eel Pond at the MBL dock adjacent to the Swope student center) was made up to SWT (6). 0.5 ml of mid-log phase, luminous *Vibrio* cultures (new isolates designated MBLB-1, Oys-1 and Oys-2 and a domesticated *V. fischeri* strain with a deletion in *luxI*; the latter obtained from P. Dunlap) were added to the SWT suspension. Following incubation overnight, 1/100 volume of  $\text{CHCl}_3$  was added and the culture examined by the phage spot assay.

**Phage purification.** Phage were purified by removing an isolated plaque from the agar with a sterile Pasteur pipette, adding 50  $\mu\text{l}$  of fresh cell culture in SWT, and incubating at rm temp for 15 min. 2.5 ml of SWT was added and the culture incubated at rm temp with aeration overnight. Chloroform (100  $\mu\text{l}$ ) was added to complete lysis.

**Host range determination and spot assays.** Rapid assays of lytic activity and host range were done by preparing a soft agar overlay (2.5 ml) of freshly grown *Vibrio* cultures (0.2 ml) on SWT. After the top agar had solidified, 10  $\mu\text{l}$  of a culture supernatant or phage lysate was applied to the agar surface. Plates were incubated overnight, or longer, at rm temp.

**Electron Microscopy.** Concentrated (0.02  $\mu\text{m}$ ) water, clarified Mitomycin C-treated culture supernatants, or potential phage lysates, were applied (8  $\mu\text{l}$ ) to carbon-coated grids, wicked semi-dry with Kimwipes after ca. 4 min, and then negatively stained with 3% uranyl acetate or 2.5% ammonium molybdate solutions (8  $\mu\text{l}$ ) directly on the grids for 1 - 2 min. Air-dried grids were examined by TEM (Zeiss 10CA) in the MBL central microscopy facility.

## Results and Discussion

### *Phage of Phototrophs*

**Lytic bacteriophages of purple non-sulfur bacteria were not isolated.** A collection of 12 purple non-sulfur phototrophic bacteria isolated from a fresh water marsh was used to screen for bacteriophages in local fresh waters. Based on their cell morphology (light microscopy), pigmentation, motility (or lack thereof), variable heterotrophic growth in the dark with oxygen, and variable hybridization to rRNA probes for the  $\alpha$  and  $\beta$  proteobacteria (see course exercises), the potential hosts likely included the genera *Rhodobacter*, *Rhodopseudomonas* and *Rhodomicrobium*. However, the strains were not definitively characterized to genus. In some experiments, the type strain *Rhodospirillum rubrum* (C. Bauer, Indiana University) was used.

Water samples screened for plaque forming units were collected from the School Street Marsh, the ditch water near the ball field at the St. Joseph's Church Bell Tower playground (BFD), Cedar Swamp Marsh, and a private ornamental pond near the Gansett-Woods neighborhood. Water was used i) directly, ii) filtered through glass fiber, iii) filtered through 0.4  $\mu\text{m}$  nylon membranes, or iv) concentrated 100-fold with 0.02  $\mu\text{m}$  supported Acrodisk filters.

Attempts were made to isolate lytic phage directly from each of the water samples processed as described above (7, 8). Initially, 20  $\mu\text{l}$  samples were spotted onto top agar lawns (PNSB or PYVS media) of the 12 phototrophic strains; the plates were then incubated



anaerobically in the light. For all but three strains, the host bacteria failed to grow on PYVS agar; only PNSB was subsequently used. No zones of clearing or plaques were observed on any of these plates. The volume of water assayed was increased to 0.1 ml, and by 100-fold concentration, and in this case potential plaques were observed from the BFD water plated with phototrophs No. 8 and 12. One or more of these plaques were plucked and incubated 4 days with strain 8 or 12. Spot assays of these plaque enrichments failed to show plaques on several hosts tested (e.g., see Figure 1). In other assays that also failed to yield plaques, 1 mM  $\text{MgSO}_4$  and  $\text{CaCl}_2$  were added to the cell-water-top agar mixture during plating.

**Apparent induction of bacteriocin by Mitomycin C.** The presence of lysogenic phage in the collection of phototrophic bacteria was examined using Mitomycin C induction. Using 18 ml capped tubes, 2 ml of a culture grown in PNSB was transferred to the same medium, to which Mitomycin C was added at a final concentration of 0.2  $\mu\text{g/ml}$ . After 3 - 5 days there was a marked reduction in the density of those cultures containing the radiomimetic agent compared to the untreated cultures. From the Mitomycin C cultures, 20  $\mu\text{l}$  were applied in spot assays to lawns of several of the 12 phototrophs. Again, obvious plaques were not observed.

Apparent bacteriocin production was observed in the Mitomycin C incubations of phototrophs 1 and 3. The inhibitory activity appeared greatest on phototrophs 1, 3 and 8 (Figure 1). Although it could be argued that these strains are just highly sensitive to Mitomycin C, phototroph 8 was most sensitive to the induced cultures of strains 1 and 3, yet 8 grew fairly well from the induced culture.

**Bacteriophage are present in local fresh water.** Concentrated (0.02  $\mu\text{m}$ ) BFD water was negatively stained and examined for phage using TEM. In several grid fields examined, hexagonal (icosahedral?) phage head-like structures were observed (Figure 2). Occasionally, less organized negatively staining structures were observed that could be other forms of phage or virus particles.

**Summary of phototroph survey.** While phage abound in fresh waters (especially polluted, nutrient-rich water), the results obtained here suggest it is not always easy to find the suitable phage-host pair. No phage of the purple non-sulfur phototrophs were isolated. However, it does appear that bacteriocin production by some strains can be induced by Mitomycin C. These bactericidal compounds, often proteins, can be useful for characterizing cell populations, exploring gene regulation, or can be purified to exploit their specific enzymatic activity (e.g., RNase, DNase, membrane depolarization). It should be noted that the phototroph isolates 1 and 8 are brown pigmented, non-motile rods in clusters, and isolate 3 is a small motile rod; these bacteriocin producers may be members of the genera *Rhodobacter* or *Rhodospseudomonas*.

Future attempts at isolating phage of the phototrophs should utilize amplification methods employing large volumes of local water sources (see below and refs. 7 - 9). Alternatively, sewage can be screened, but it may not yield phage relevant to the generally unpolluted water bodies of the region. Nonetheless, such phage would be of potential genetic and ecological interest. Waterbury (10) has documented the natural adaptation of ocean cyanobacterial populations to phage resistance; resistance of co-occurring hosts to phage may account for the inability to detect plaque-forming phage for the natural *Rhodospirillaceae* isolates.

In one study (9) 16 different virulent bacteriophage types of *Rb. capsulata* were isolated. All were isolated from sewage and displayed differing plaque morphologies and host ranges. Although none of these lytic phages were able to carry out transduction, further reports on molecular biology or ecological significance of these phages is not apparent. However, other temperate phage have been isolated and more thoroughly characterized (11-13). Currently, the "gene transfer agent" (reviewed in 14 and 15) is the only phage-type gene transfer system (unidirectional) used in the Rhodospirillaceae, and apparently only in *Rb. capsulata*. Further characterization of new and previously isolated phages of the phototrophic purple non-sulfur bacteria appears warranted, particularly in light of the broad ecological distribution of these bacteria (3), and the complete genome analysis underway for *Rb. capsulata*. (16).

### **Phage of Luminous Vibrios**

**Direct screening of sea water did not yield vibriophages.** Sea water samples (obtained almost exclusively from Eel Pond, Woods Hole) were treated by filtration essentially as described above for screening for viruses of phototrophs. Three environmental isolates of luminous *Vibrios* were used in lawns as indicator strains on SWC or SWT plating media. In spot or whole plate assays, no plaque forming units were detected. The presence of 1 mM  $\text{CaCl}_2$  and  $\text{MgSO}_4$  had no noticeable affect on cells nor did it result in phage detection. A somewhat larger volume of cell culture and water sample (0.5 ml of each), incubated at rm temp for 15 min prior to the addition of 2 ml of SWT top agar and plating, also failed to yield phage. Both Eel Pond and Cape Cod Bay sediment/water (from a depth of 20 m) were used. Concentrated (0.02  $\mu\text{m}$ ) Eel Pond sea water treated in this manner was also unproductive.

Freshly shucked whole oyster was homogenized with sterile SWC (20 g:40 ml) and the filtered (0.4  $\mu\text{m}$ ) homogenate spotted directly onto the environmental isolates. Alternatively, 0.1 ml of cells and homogenate were mixed, plated and again no plaques were observed.

**Mitomycin C and heat shock failed to induce plaque forming phage.** Three environmental *Vibrio* isolates were incubated overnight (fresh inocula) in 2 ml SWC containing Mitomycin C at 0.2  $\mu\text{g}/\text{ml}$ . The cultures exhibited reduced growth or substantial clearing. When spotted onto SWC top agar lawns of all three *Vibrio* strains, no plaques or clearing was observed. A comparable experiment was conducted, again without interesting result, where fresh cultures were incubated at 50°C for 7 min, then 0.1 ml plated directly into top agar. Temperate prophage apparently were not induced, or they were unable to infect the three indicator strains.

The heat shock-treated culture of the oyster gut *Vibrio* (Oys-2) was filtered (0.4  $\mu\text{m}$ ), negatively stained and examined by TEM. Possible phage-like particles, spherical and lacking the head and tail structure typical of the Myoviridae were observed (data not shown).

**Co-culture amplification of sea water yields vibriophage.** The amplified Eel Pond sea water culture (see Materials and Methods) was spot assayed (40  $\mu\text{l}$ ) on lawns of the four *Vibrio* strains used for seeding. Plaques or growth inhibition was observed on the lawns of MBLB-1 and Oys-2 cells, but not on lawns of Oys-1 and *V. fischeri* ( $\Delta luxI$ ). Three

isolated plaques were purified from each plate as described above. Therefore, two plaque-forming phage appeared to be isolated on two separate *Vibrio* hosts.

**Phage and bacteriocin host range.** Although lysates of  $\phi$ MBL-1 could be titrated (ca  $5 \times 10^6$ ), Oys-2 lysates failed to yield plaques beyond a 1/10 dilution (Figure 3), suggesting bacteriocin, rather than phage, was responsible for the turbid "plaque" morphology. Therefore, cells from the centers of cleared zones were streaked; isolated colonies were then picked onto lawns of the four *Vibrio* strains. Figure 4 (bottom two rows) shows the small halo of clearing surrounding the colonies derived from the " $\phi$ Oys-2 plaques" when these cells are placed on a lawn of Oys-2 cells. Three culture supernatants of " $\phi$ Oys-2 lysates" (second row) inhibit Oys-2 growth. Therefore, bacteriocin production, active only against Oys-2 cells, has been detected.  $\phi$ MBL-1 clears the MBLB *Vibrio* strain significantly, and causes turbid clearing of Oys-2. It does not appear to infect the other two vibrios. Colonies from the centers of plaques do not produce halos of clearing on any of the indicator strains. Therefore these appear to be bonafide phage-resistant, non-bacteriocin producing derivatives.

**TEM reveals phage-like particles without elaborate tails.**  $\phi$ MBL-1 lysates that were untreated, filtered (0.4  $\mu$ m), or incubated with MBLB *Vibrio* cells were negatively stained and examined by TEM. Phage head-like particles were apparent, but clear, elaborate tail structures were absent (data not shown). Although preparation of this marine vibriophage for TEM has not been optimized, the particles observed suggest the phage is a member of the viral family *Podoviridae*.

**Summary of vibriophage isolation.** Isolation of a few phages of the luminous *Vibrios* has been reported (6, 17), with progress toward developing a system to transduce genes among *V. fischeri* strains (6). Characterization of additional phage will enhance the genetic analysis of *Vibrio*, but are worthy of study in their own right. Gene regulatory mechanisms have not been extensively studied in the marine vibrios and the vibriophages should have something to contribute to that area. The population dynamics of luminous vibrios, including "quorum sensing", particularly as it relates to cell surfaces and receptors (18), can be addressed using phage as a biological probe. Recent studies on colonization by luminous symbionts suggest that phage may be helpful in characterizing relevant surface components of the participating *Vibrios* (19; K. Viseck, pers. comm.).

Amplification by seeded cultures, using more than one strain as an inoculum, was shown to be a useful approach for isolating vibriophages. In this single example, more than one "bacteriolytic" activity was identified, so potentially multiple phage can be enriched in a single amplification culture. In less than two days, at least one lytic phage,  $\phi$ MBL-1, was isolated by the simple seeded amplification procedure.

## Acknowledgments

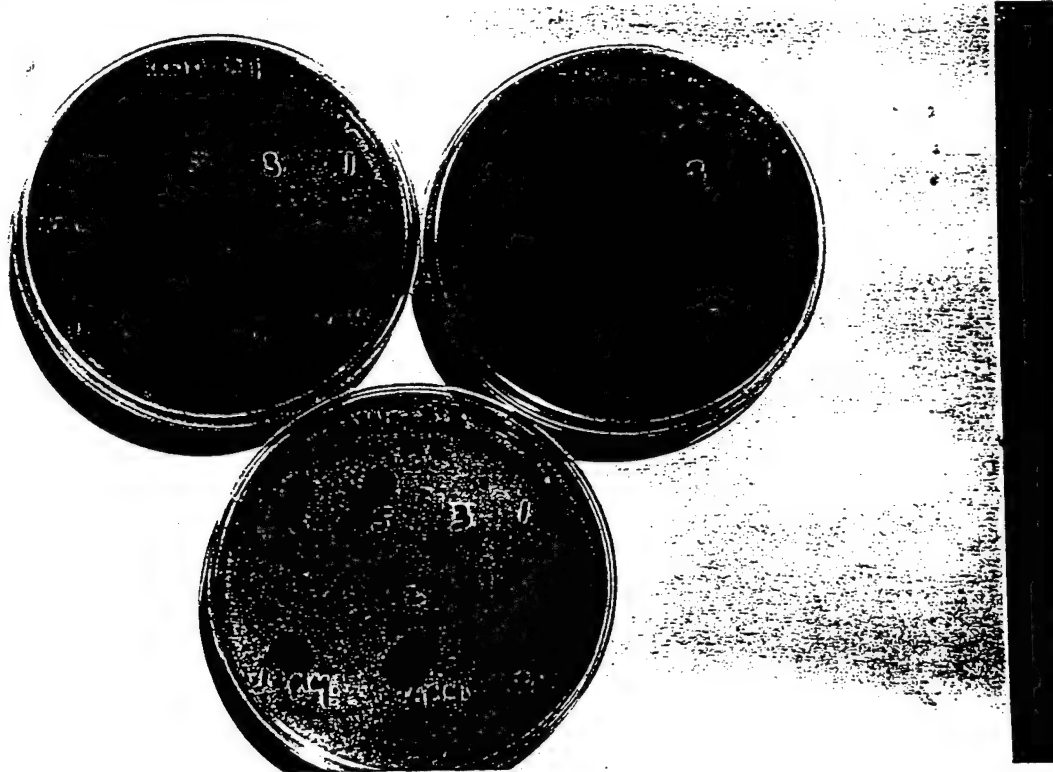
I thank the Course Directors, MBL, ONR and NCSU for providing me the opportunity to participate in this wonderfully fulfilling and intellectually stimulating program. My family (Sherri, Ethan and Lindsey) came along for the fun of it all and diligently tolerated my brief appearances, usually to partake in a meal and only to quickly disappear. Tom Pitta and Liz Sherwood introduced me to the simplicity of negative staining, while Tom set me up to drive the TEM. Jorg Overmann did a great job familiarizing me with the phototrophs, and ditto for Paul Dunlap and Ned Ruby on the luminous *Vibrios*. Karen Viseck provided kind email discussions on media and vibriophages.

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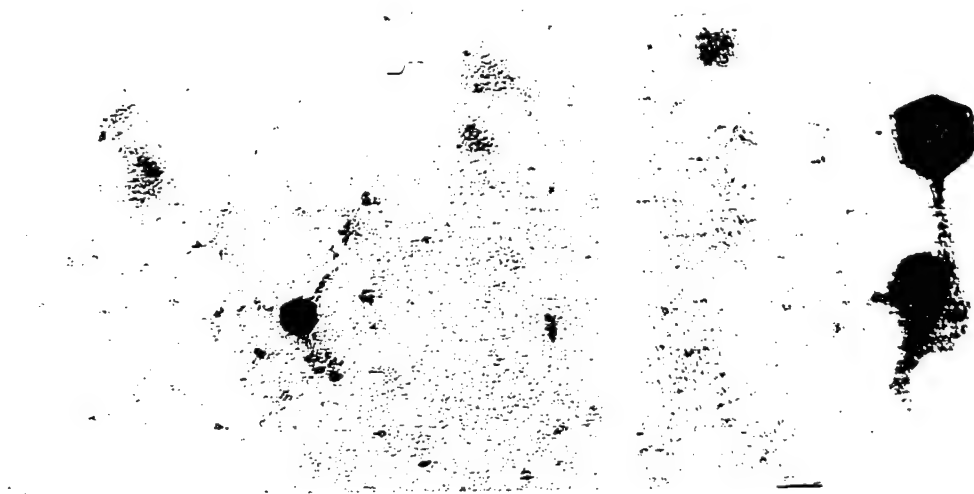
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**Figure 1.** Bacteriocin production by three purple non-sulfur phototrophs treated with Mitomycin C.

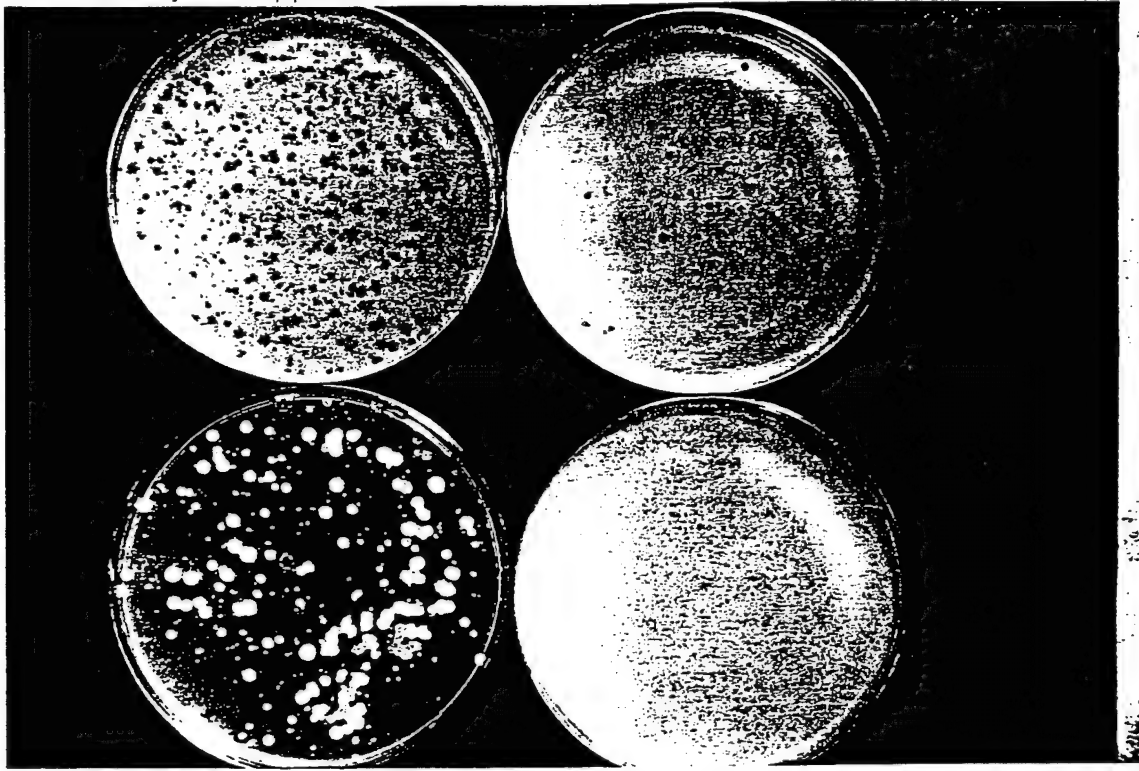


**Figure 2.** TEM of negatively stained phage particles from ditch water (BFD). 50K and 100K magnification of two different particles, with a 2X photographic enlargement.

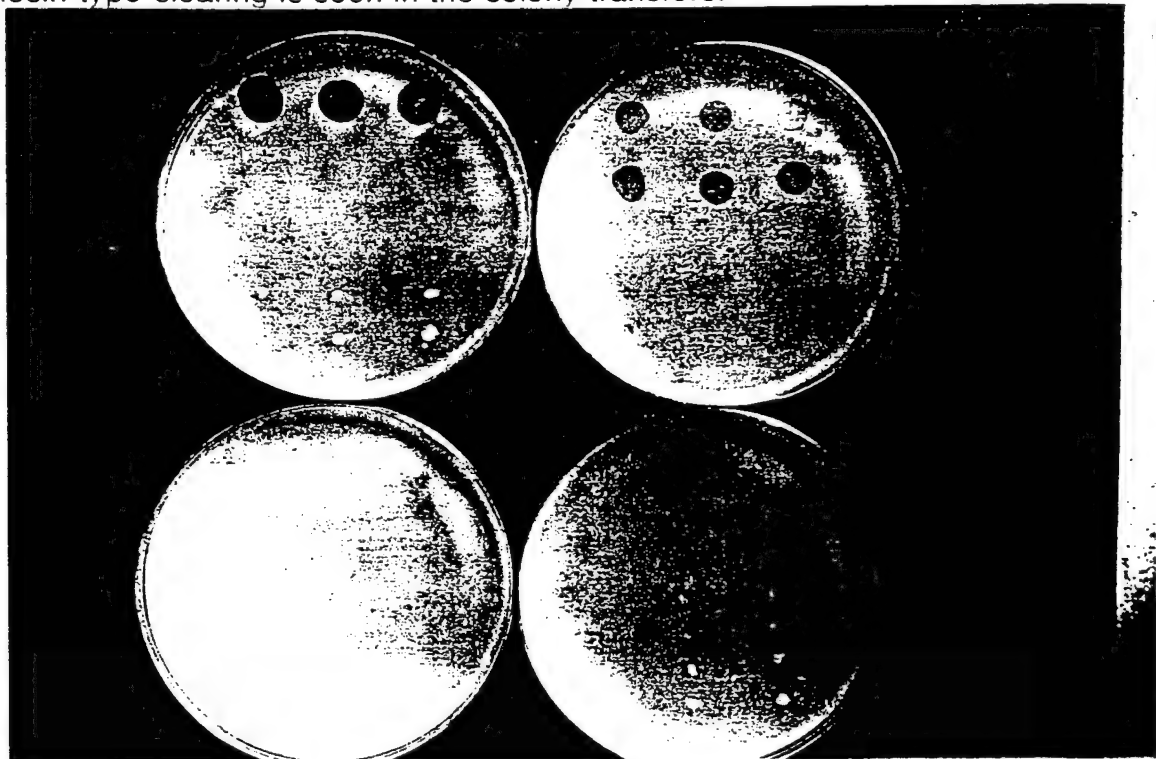




**Figure 3.** Titers of lytic activities. Top row is a lysate of  $\phi$ MBL-1 (prepared on strain MBLB-1) diluted to  $10^{-7}$  and  $10^{-8}$  and plated on MBLB cells. The bottom row is a "lysate of  $\phi$ Oys-2" (prepared on Oys-2 cells) diluted  $10^{-1}$  and  $10^{-2}$  and plated on Oys-2 cells. Phage and bacteriocin activity are apparent.



**Figure 4.** Host range of phage and bacteriocin activities. Cell lawns are: *top row*, MBLB, Oys-2 (l to r); *bottom row*, Oys-1 and *V. fischeri*. For lysate spot assays, the top row on each plate is 3 separate lysates of  $\phi$ MBL-1 and the second row is 3 separate preparations of " $\phi$ Oys-2". Below the phage are colony transfers of potential lysogens or resistant cells from lysis zones of  $\phi$ MBL-1 (two rows) and " $\phi$ Oys-2" (bottom two rows). Note that  $\phi$ MBL-1 has a broader host range and substantially clear MBLB cells.  $\phi$ Oys-2 affects only Oys-2 cells, and bacteriocin-type clearing is seen in the colony transfers.



Tracing the Development of Natural Biofilms with In Situ Hybridization and  
Scanning Confocal Laser Microscopy

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## Introduction

It is by now axiomatic that the diversity of microorganisms in the environment has barely begun to be represented in laboratory culture. The reasons for this are manifold, yet one important cause may be the fact that the culturing environment used in most laboratories is primarily liquid-based. In nature, however, the majority of bacteria are attached to surfaces. Studies have shown that many more types of cells are able to develop in biofilms than in the bulk fluid of surrounding ecosystems (1). Biofilms are omnipresent, found everywhere from the surfaces of rocks in streams to the surfaces of our teeth. While the importance of biofilms in the environment has long been appreciated (2), surprisingly little attention has been paid to characterizing their microbial communities.

Recent advances in molecular biology and imaging technologies have made this type of characterization possible for the first time. Molecular and two-dimensional microscopic identification of sulfate-reducing bacteria in "artificially" created biofilms has been performed (3), as has three-dimensional microscopic examination of an artificial community of *Pseudomonas fluorescens*, *Pseudomonas aeruginosa* and *Vibrio parahaemolyticus* (4). To date, however, these approaches have not been combined. The main goal of this project was to employ in situ hybridization in conjunction with scanning confocal laser microscopy (SCLM) to trace the development of microbial populations in natural biofilms. A secondary goal was to qualitatively determine which cells required the biofilm environment for growth, and which could be cultured on agar or liquid media.

## Materials and Methods

### I. General Approach

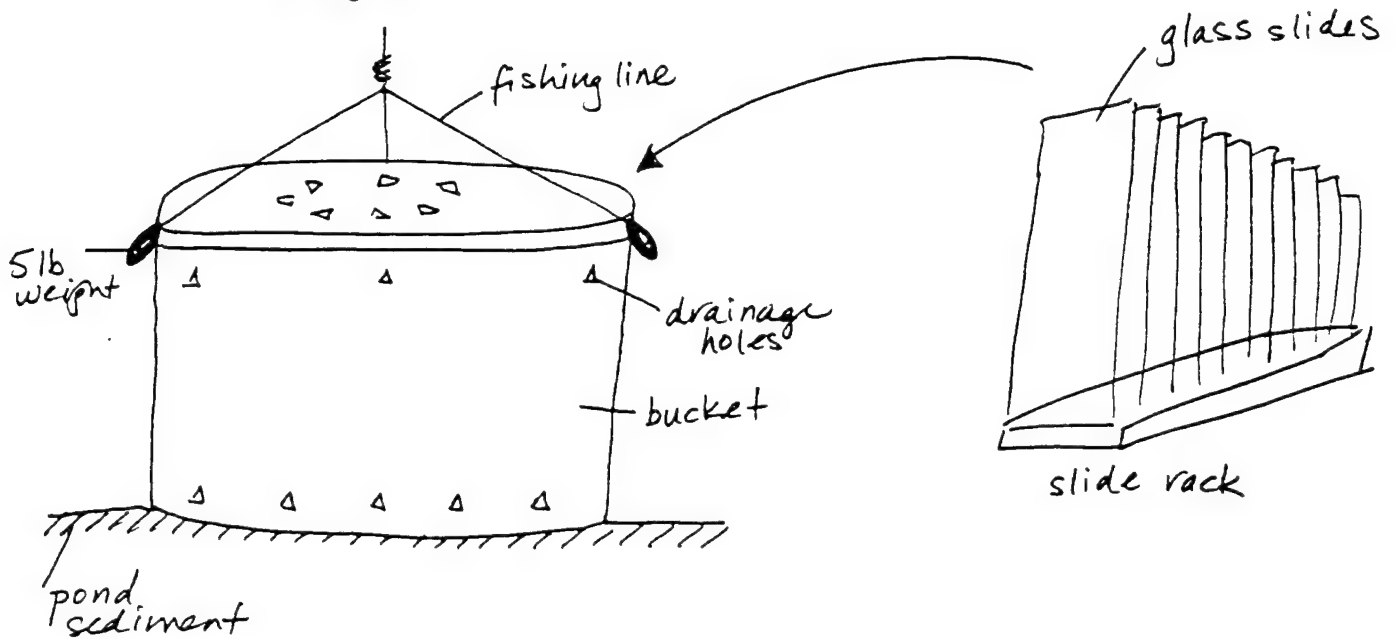
Biofilms were developed in two aquatic environments: a freshwater and a brackish pond near Gansett Beach. A covered bucket which contained glass slides for biofilm attachment was lowered into each pond at the beginning of the experiment and left to develop for two weeks. At regular intervals (days 1, 6 and 14) slides were retrieved from the buckets and prepared for microscopy and in-situ hybridization. Agar and liquid cultures of the biofilm bacteria were obtained by imprinting the slides onto a variety of plates (Yeast Extract Glucose, YEG; Case Amino Acids, CAA; and Sea Water Complete, SWC) on day 11, and picking single colonies for restreaking and/or inoculation into liquid media on day 13. Initially, plates were prepared from both freshwater and brackish biofilms. Due to time and resource constraints, however, only the brackish samples in CAA and SWC media were maintained for further study. Five representative yet apparently different colonies were selected from the SWC imprint, and four were selected from the CAA imprint. These colonies were restreaked several times, and transferred into appropriate liquid media.

### II. Construction of Sampling Buckets

A. Materials: white plastic bucket; 5lb fishing weights, fishing hooks, fishing line; glass slides, slide holders.

B. Assembly: Drilled holes in the top, bottom and sides of the bucket to allow water to drain; hung 5lb weights on 3 hooks and fastened to the bucket; sewed two slide holders onto bottom of bucket with fishing line; placed 30 slides in the holders (15 slides each); lowered into the ponds.

C. Diagram:



III. Microscopy

A. Epifluorescence/Phase-Contrast: Slides were retrieved from the buckets and inspected for autofluorescence on a Zeiss Axioskop prior to fixation. No preparation was necessary.

B. SCLM: Slides were retrieved from the buckets and placed in a humid environment (wet Kimwips in a slide holder) to preserve the structural integrity of the biofilm. Slides were stained with acridine orange (.01% in distilled H<sub>2</sub>O) for 1 minute, covered with a coverslip, and mounted on a Zeiss LSM 410 Laser Scanning Confocal Microscope while wet.

C. SEM: Scanning electron microscopy was used to visualize the biofilm surface in three-dimensions. Slides were prepared by fixation in 2% glutaraldehyde for 30 minutes, followed by two 15 minute washes in .1M

sodium cacodylate. An ethanol dehydration series followed, where slides were suspended in 50, 70, 85, 95 and 100% ethanol for 10 minutes each. The slides were cut down to 1 cm<sup>2</sup> areas and dried in a Tousimis Samdri 780-A critical point drier. A Tousimis Samsputter 2a was used to coat the slides with gold/palladium (60:40%), after which they were examined with a JEOL JSM-840 scanning microscope operating at 15 kV.

IV. In-Situ Hybridization: Two different sets of slides were prepared for hybridization: the first came directly from the ponds where the biofilms originated, the second was prepared from cells grown on agar plates. Undipped slides were used for the first set, as the organic matrix of the biofilm served as a natural adherent and application of gelatin would have radically altered the surface chemistry of the slide. Because silicates and alumino-silicates comprise a large fraction of sedimentary rocks (5), such alteration would have been undesirable: plain glass slides more accurately represent natural surfaces for microbial attachment. The pond slides were fixed for 1-2hrs in a 37% formaldehyde: 100% ethanol solution (ratio 10:90), freshly made prior to use. Three washes in distilled water (15 minutes each) followed, and the slides were air-dried overnight. The second set of slides was prepared from the five colonies that had been cultivated on SWC plates. A loopful of cells was suspended in PBS, and diluted 100-fold. 15 µl of this solution was applied to the wells of gelatin-dipped slides, which were air-dried overnight.

The same probes were applied to all of the slides. These included: Universal A, ENT, High G+C, Low G+C, Alpha, Beta, and Flavo. A negative control was done with the hybridization mixture alone, and positive controls were done in conjunction with the rest of the class. Slides containing High G+C, ENT, and Universal A probes were incubated overnight at 37 °C. All others were incubated overnight at 45 °C.

V. Gram Stain and Oxidase Test: The Gram stain was determined with a Gram Stain Set by Fisher Diagnostics. Samples were prepared from the five brackish SWC colonies, and compared to a positive (lactic-acid bacterium) and negative (psuedemonad) control. The Oxidase test was performed on the same samples with a Spot Test Oxidase Reagent by Difco Laboratories.

### Results and Discussion

Phase-contrast microscopy revealed that a variety of microbes adhered to the slides as early as day 1. Figure 1 and Figure 2 depict the most commonly seen organisms from the brackish and freshwater ponds. In general, more diversity was seen on the brackish slides.

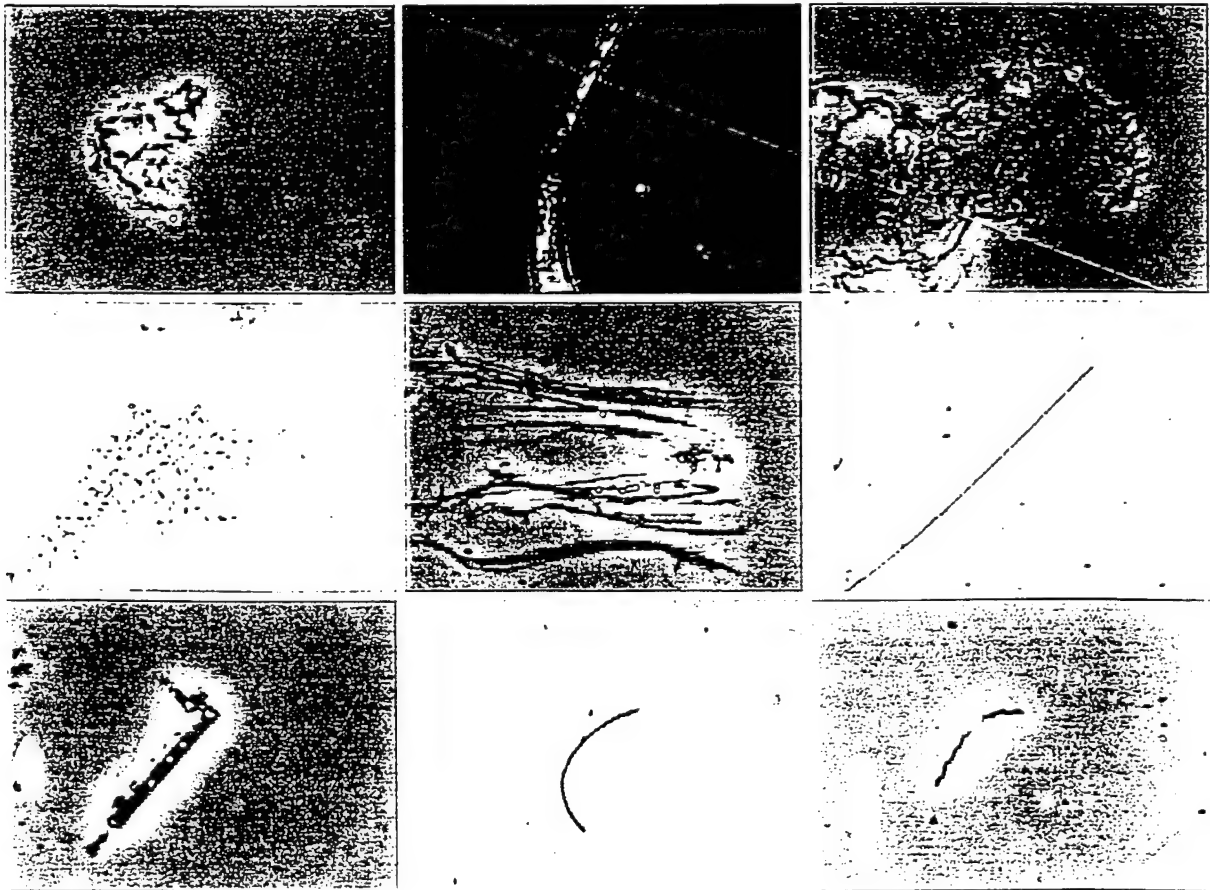
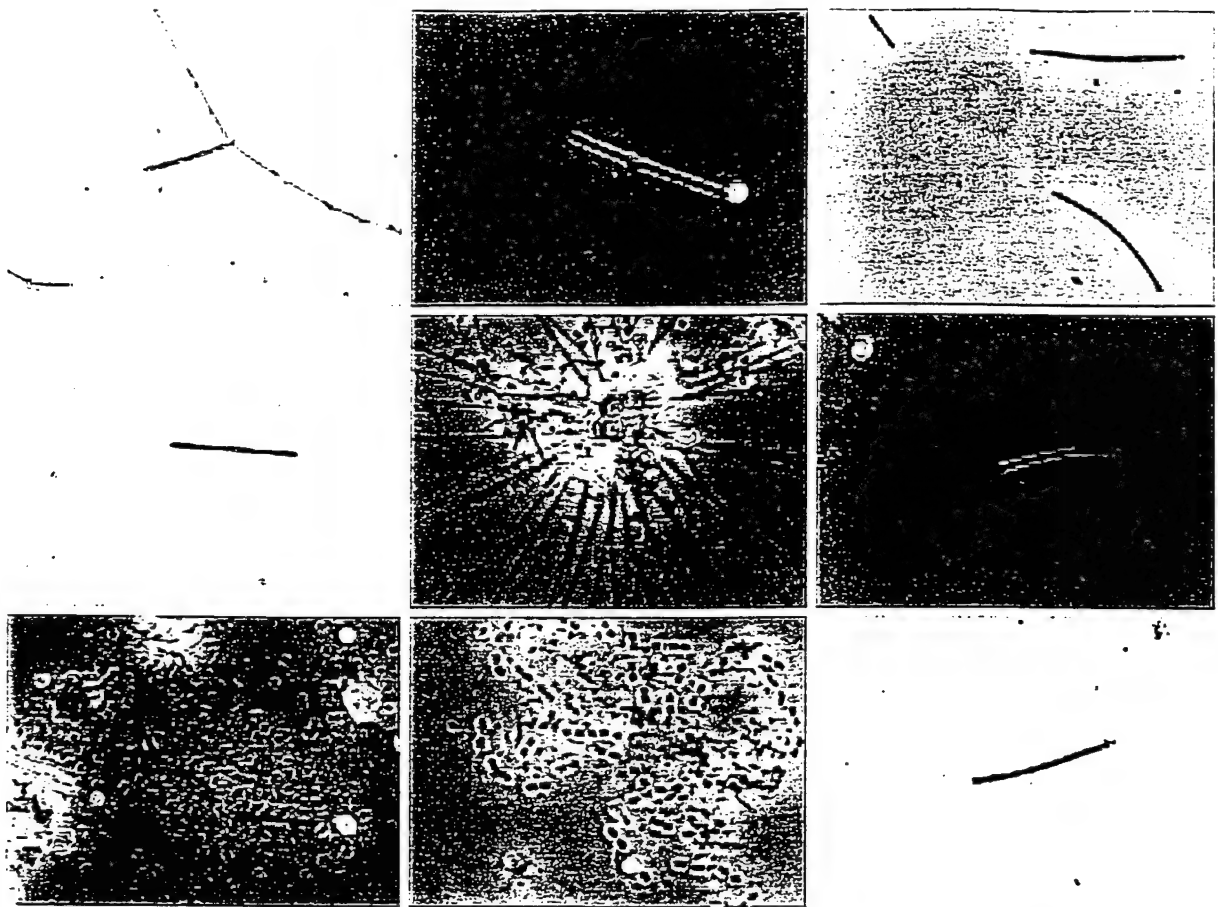


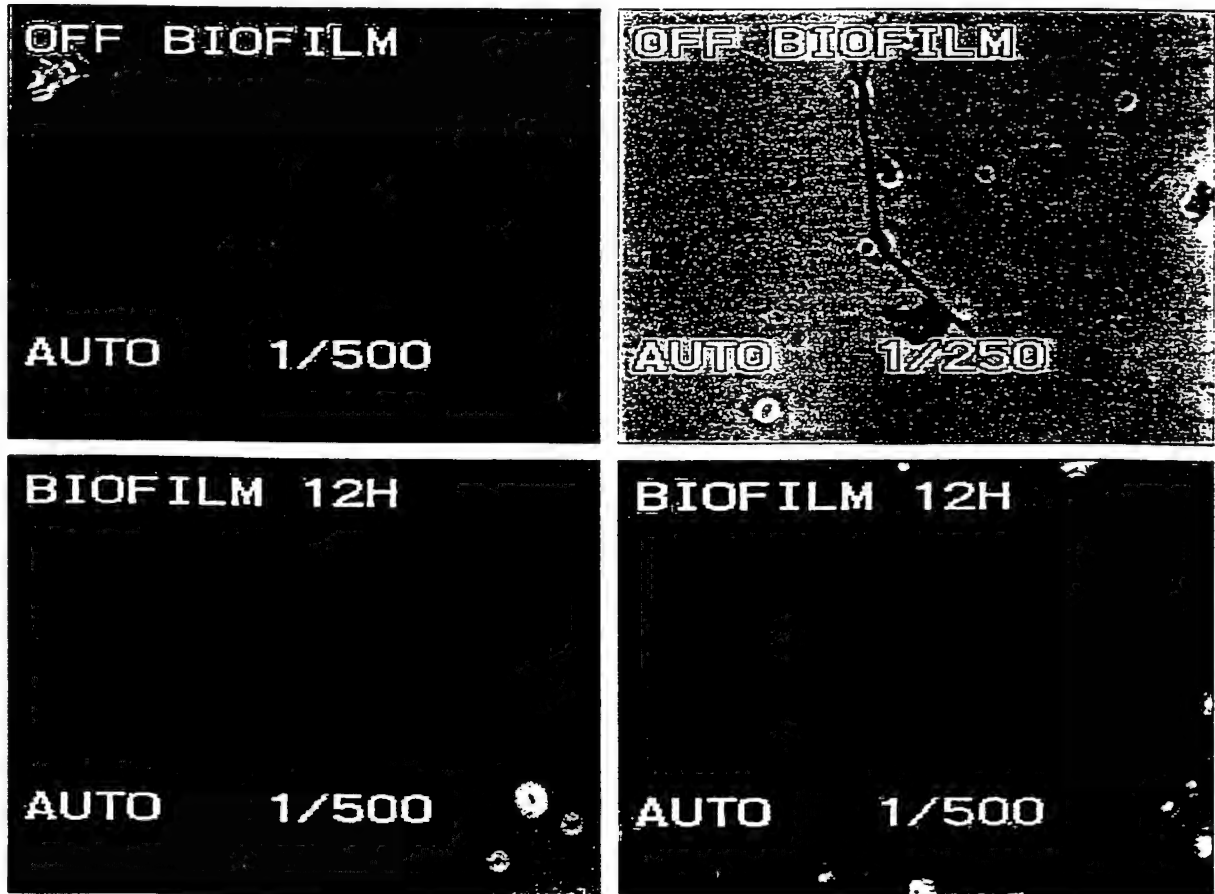
Figure 1: Brackish Biofilm Organisms  
day 1

Figure 2: Freshwater Biofilm Orga. day 1



A slime layer (biofilm) appeared to select for the growth of spirochete bacteria on the brackish slides; these bacteria were not observed on "dry" regions of the slide, which favored long rod-shaped organisms (Figure 3).

Figure 3: Organisms visible "on" Biofilm and "off" Biofilm



A variety of colonies developed from the imprints of the two biofilms. The largest diversity was obtained from the brackish sample growing on SWC media. Table 1 details the types of colonies picked and restreaked on YEG, CAA and SWC plates for both freshwater and brackish samples.

Ref. #	Colony description	SWC b	YEG b	CAA b	YEG f	CAA f
0	coral/peach; shiny	X				
1	bright orange; shiny	X				
2	chalky yellow; mucoid	X		X		
3	chalky lt. orange; mucoid	X				
4	white/coffee; mucoid	X	X	X	X	X
5	white lacy spread	X	X	X	X	X
6	white highly textured	X		X		X
7	<i>P.fluorescens</i> type					X

**Table 1:** Most common colonies from biofilm imprints on plates. X indicates presence of colony on plate, "b" stands for "brackish biofilm," "f" stands for "freshwater biofilm."

The media was selective: seven different colony-types grew up on SWC, but only four on CAA and two on YEG. Apart from the fact that the greatest variety of colonies appeared on the SWC plate, the colonies it contained were more "manageable" than similar colonies on YEG or CAA plates. Growth was particularly "messy" on YEG: the bacteria rapidly spread over the entire surface of the plate. Time and resource constraints prohibited detailed examination of each of these organisms, so the five most representative colonies from the SWC brackish plate were selected for further study. Table 2 describes their appearance under the microscope, their Gram type, whether they were oxidase positive, and how well they grew in liquid media after a period of 3 days. See Appendix for photos.

Ref. #	Description	Gram type	Oxidase presence	Growth after 3 days in liquid
1	short rods, some motile	-	++	+++
2	spore formers, rods	-/(+)	+	++
3	non motile rods	-	++	++
4	long rods, non motile	+	++	+++
5	mixed culture: long non motile rods, short motile rods	-	+	++

**Table 2:** Characteristics of five brackish SWC colony-types.

In-situ hybridization was used to roughly place these colony-types in phylogenetic classes, as seen in Table 3.

Ref. #	Hyd. mix	Univ. A	ENT	High G+C	Low G+C	Alpha	Beta	Flavo
1		X	X					
2		X	X		~	~	~	
3		X					X	
4		X			X		~	
5		X				~	~	X

**Table 3:** In-situ hybridization of five brackish SWC colony-types. "X" indicates where fluorescence was observed. "~" indicates weak fluorescence. No "X" in the "Hyd. mix" column means there was no autofluorescence.



The lack of a unique signal for colony-types 2, 4 and 5 may be due in part to unspecific binding of the probes (particularly in the case of the Beta probe; possibly storage conditions were not sufficiently stringent), and in part to the fact that the colonies were not necessarily pure and therefore could hybridize to more than one probe. The results of the Gram-stain also help explain the results (Table 2). In the case of colony number 2, the Gram stain was variable--mostly negative, but partly positive. This ambiguity sheds light on why a weak signal was detected for "Low G+C." Similarly, the clear Gram positive stain of colony type 4 favors its identification as "Low G+C."

Regrettably, no results were obtainable for in-situ hybridization on the slides from the ponds. This was due to the lack of sufficient cell density to get a detectable signal on the Zeiss Axioskop, and probably exacerbated by the spreading of the probe on the slide which further weakened its signal. SCLM was not helpful either, as the thickness of the biofilm was too small to warrant its usage. While fluorescence was observed with the acridine orange stain, three-dimensional reconstructions of the biofilm were impractical. Scanning electron microscopy proved to give the best three-dimensional picture of the biofilm, and while it was only qualitative, it clearly showed that a variety of morphotypes were present within the biofilm that were not cultured on the plates (see Appendix for photos).

## Conclusions

Combining in-situ hybridization and SCLM potentially could be an effective approach to expanding our knowledge of the biofilm community. If this is to succeed, however, more time is necessary for the development of the biofilm. The results described here suggest that freshwater and brackish pond biofilms do not become thick enough in two weeks to make direct in-situ hybridization and SCLM viewing practical. Nevertheless, SEM and phase-contrast images clearly show that a large diversity of organisms can

attach to surfaces within a short period of time in these environments, and that this diversity is difficult to capture through traditional enrichment techniques. Because of the weak signals on the pond slides, information regarding the evolution of the microbial communities over time was impossible to obtain. What is evident, however, is that in-situ hybridization can be used for preliminary phylogenetic identification of the predominant *culturable* organisms.

If this experiment were to be repeated, several improvements could be made:

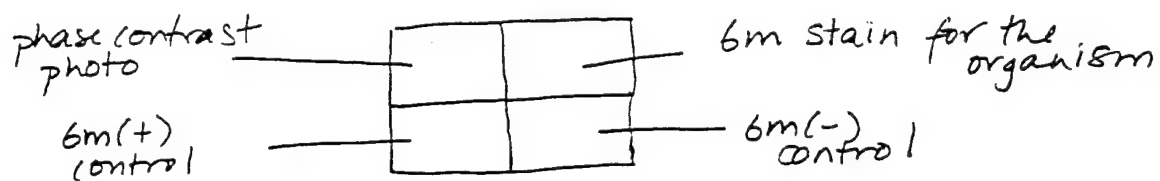
- 1.) Glass slides could be procured that contained sunken wells. This would improve the odds of in-situ hybridization succeeding in the pond samples, as the probe could be contained in later application, and the signal thereby enhanced.
- 2.) Slides could sit for a longer period of time so the biofilm could become thicker. Alternatively, one could get a sample of a mature biofilm--for instance, one lining the insides of a pipe.
- 3.) Different and more types of media could be tried to bring as many organisms as possible into culture.
- 4.) Changes could be made in the in-situ hybridization procedure to improve the stringency of probe binding.

### **References**

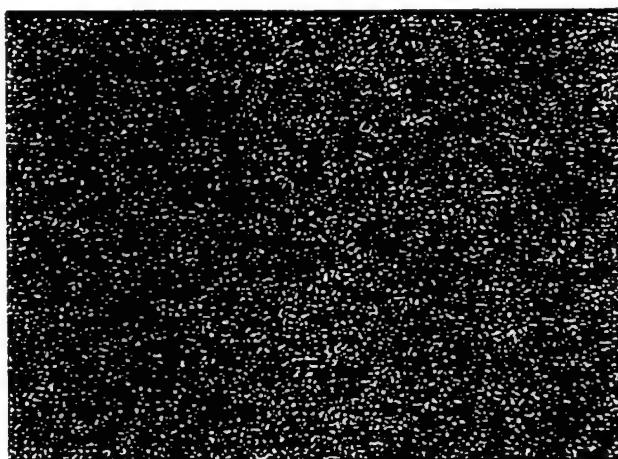
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## Appendix

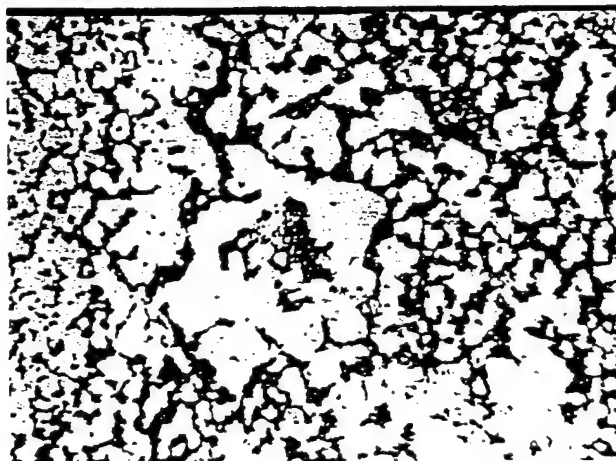
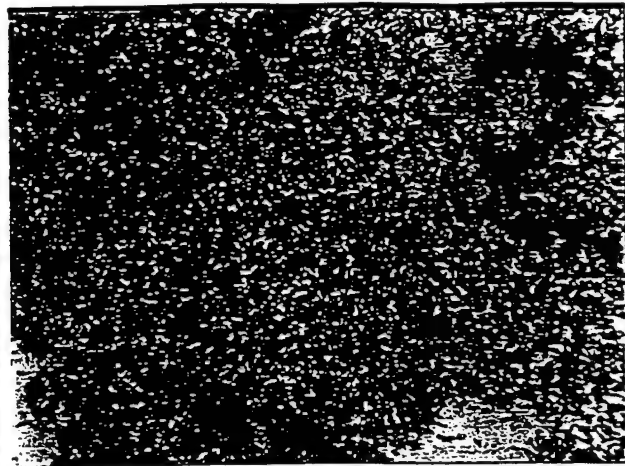
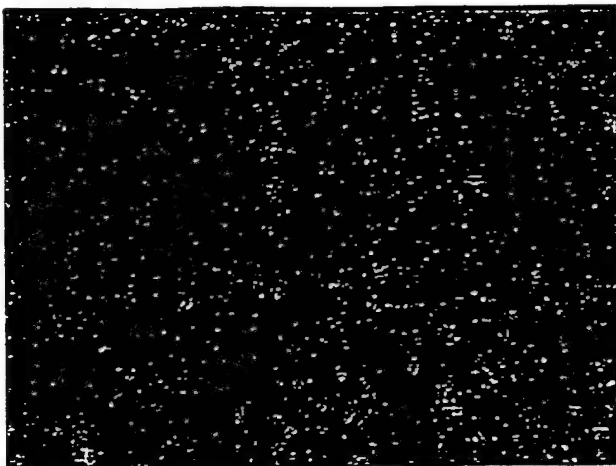
Gram stains for the 5 SWC colony-types follow.



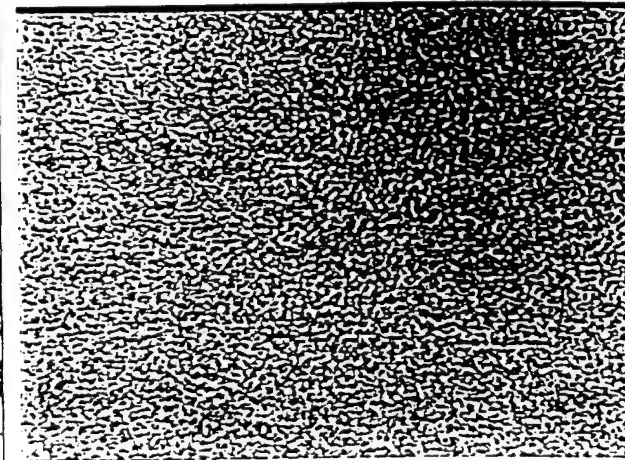
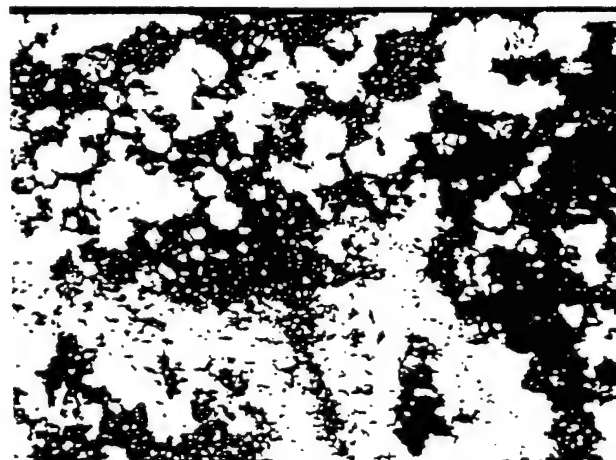
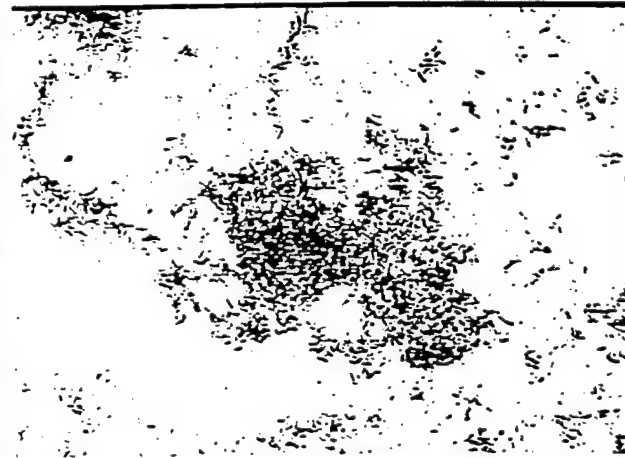
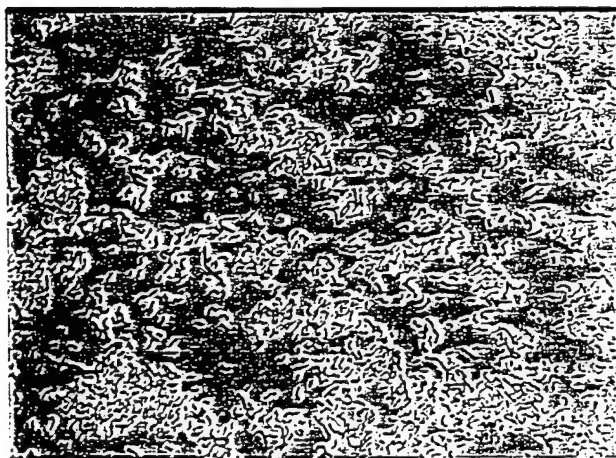
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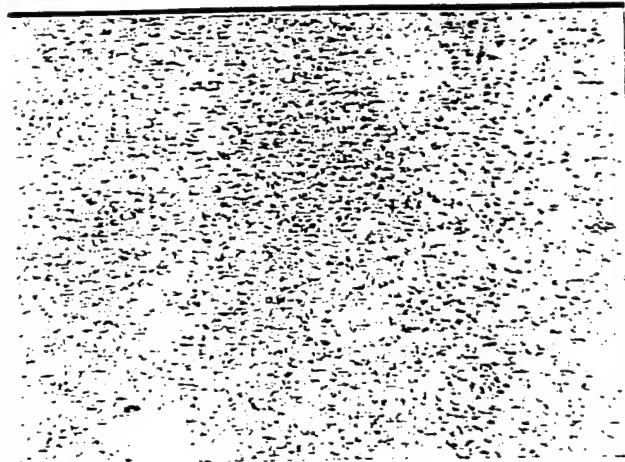
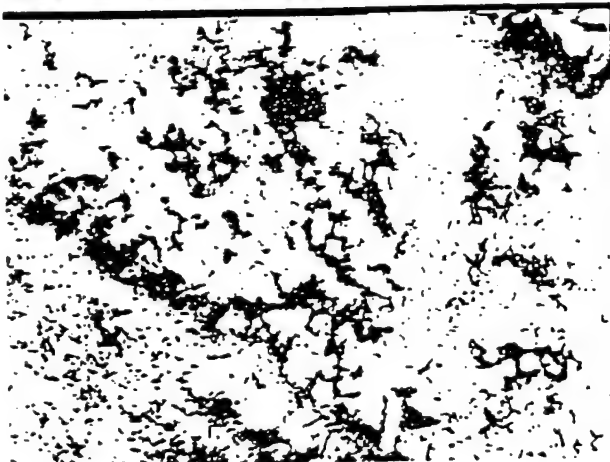
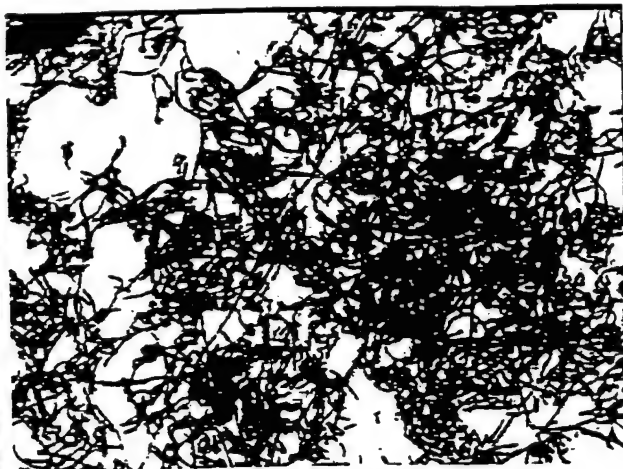
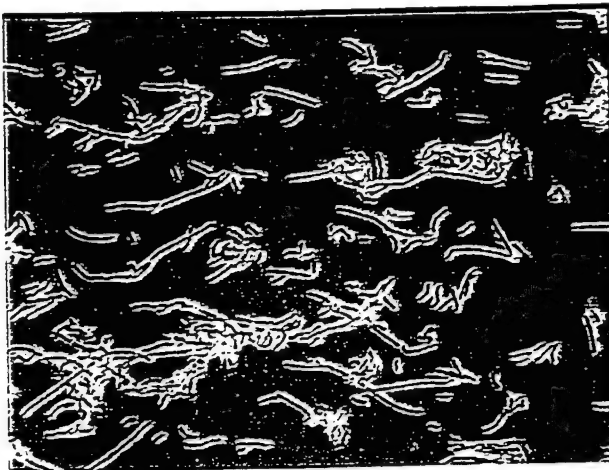
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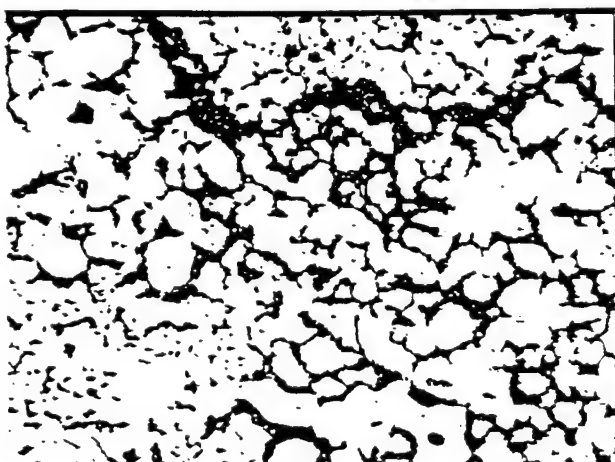
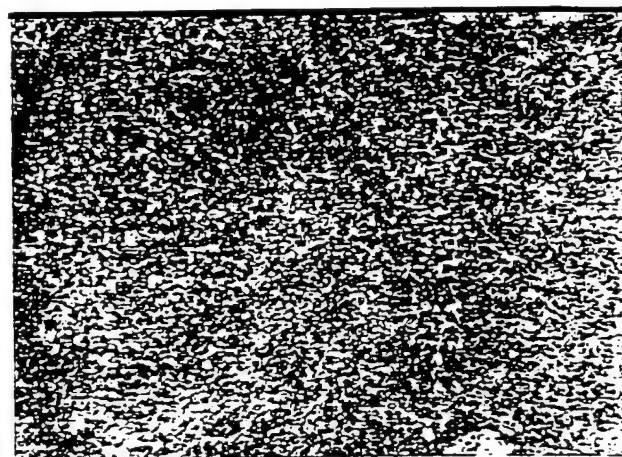
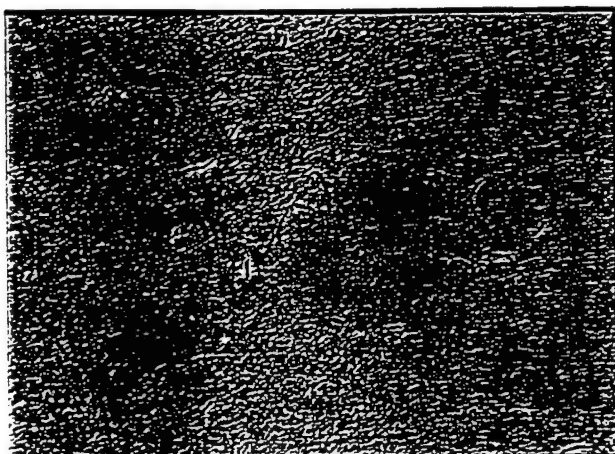
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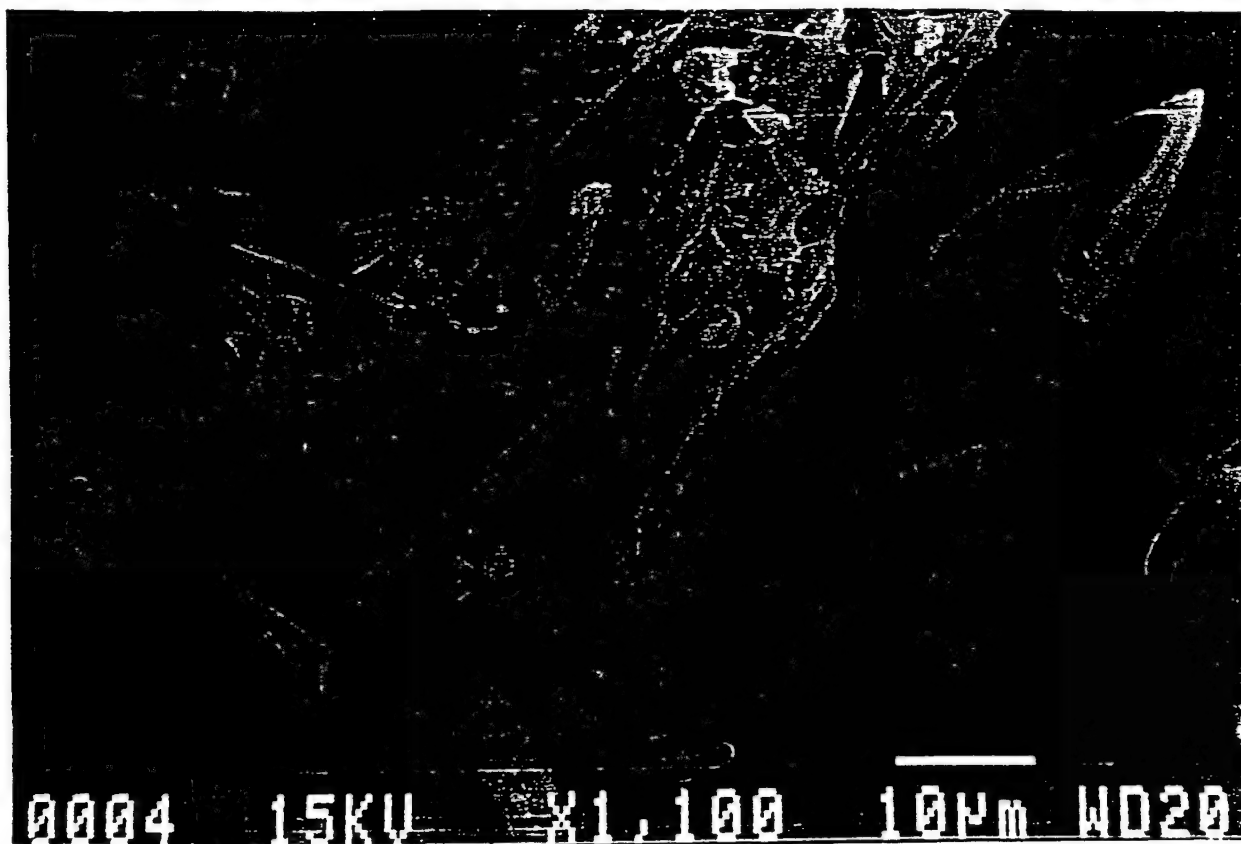
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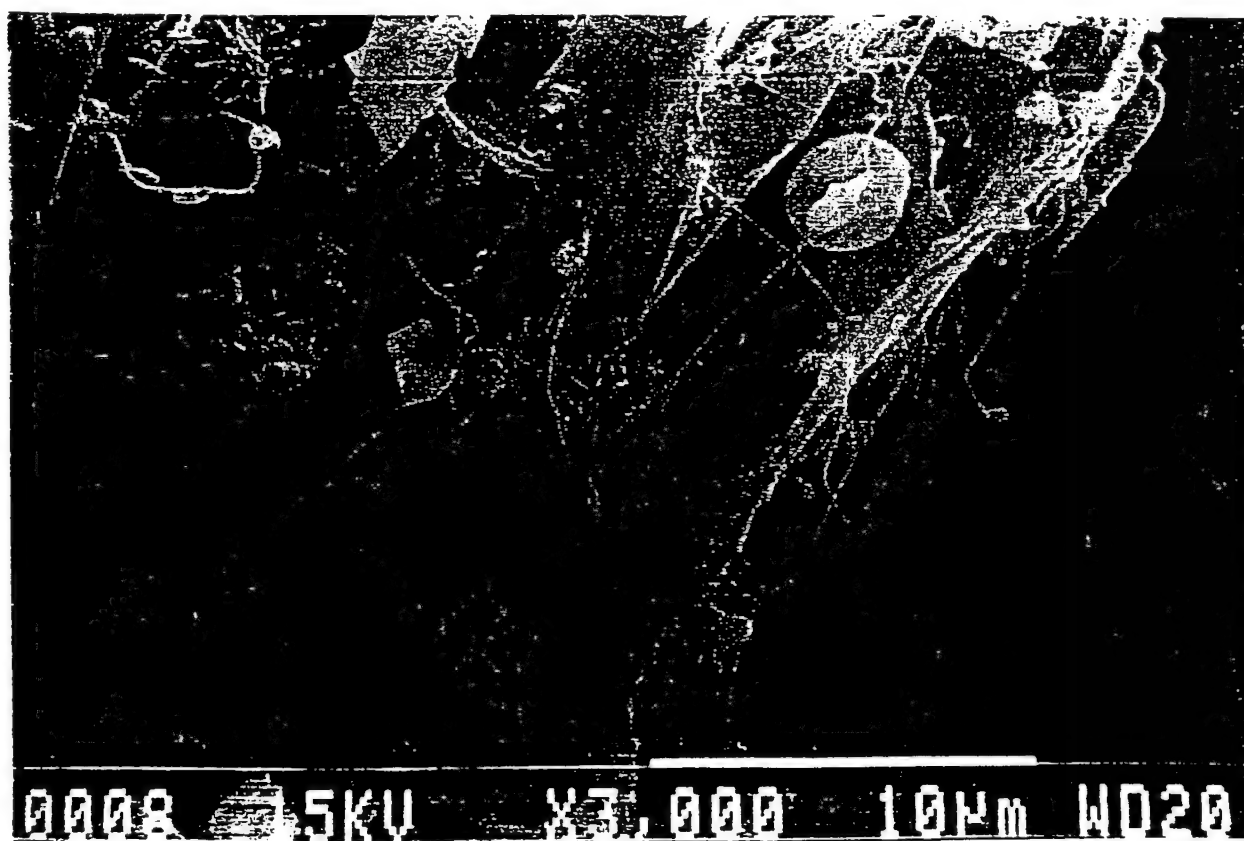
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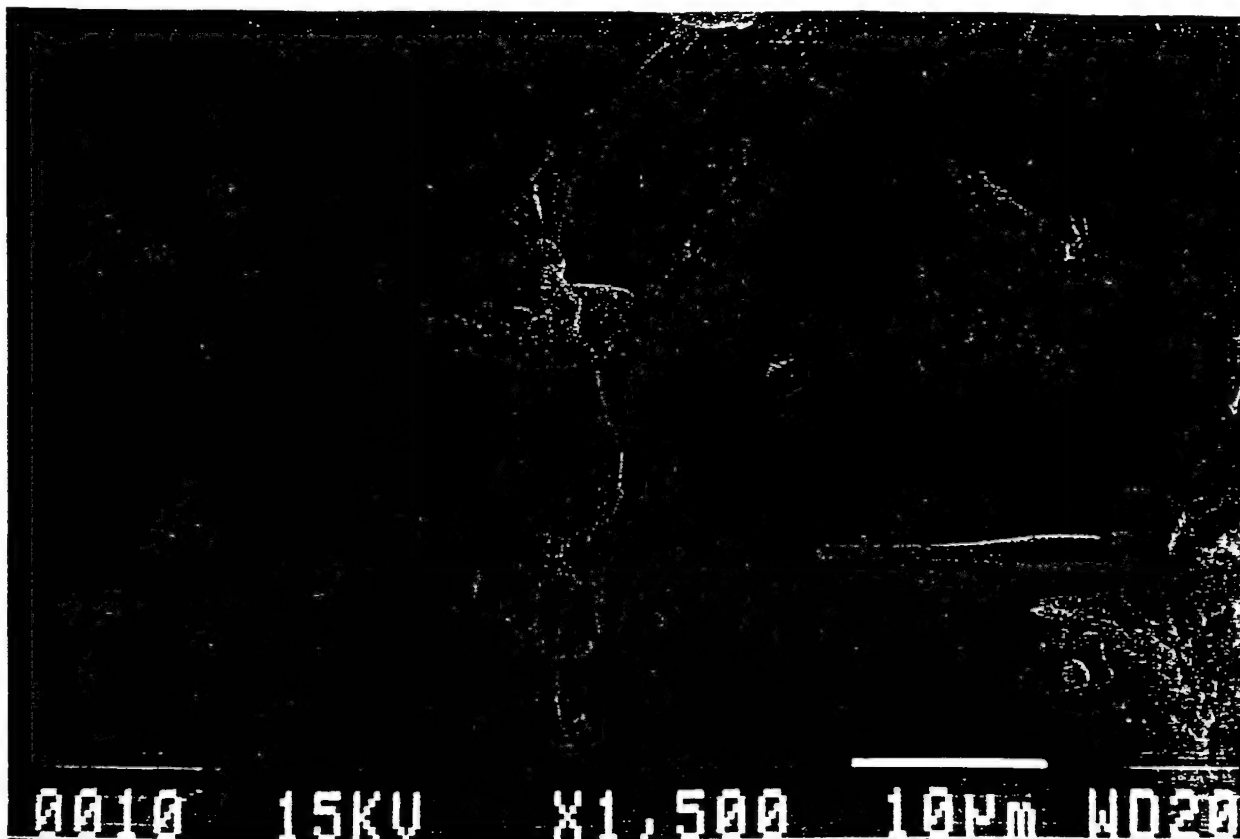




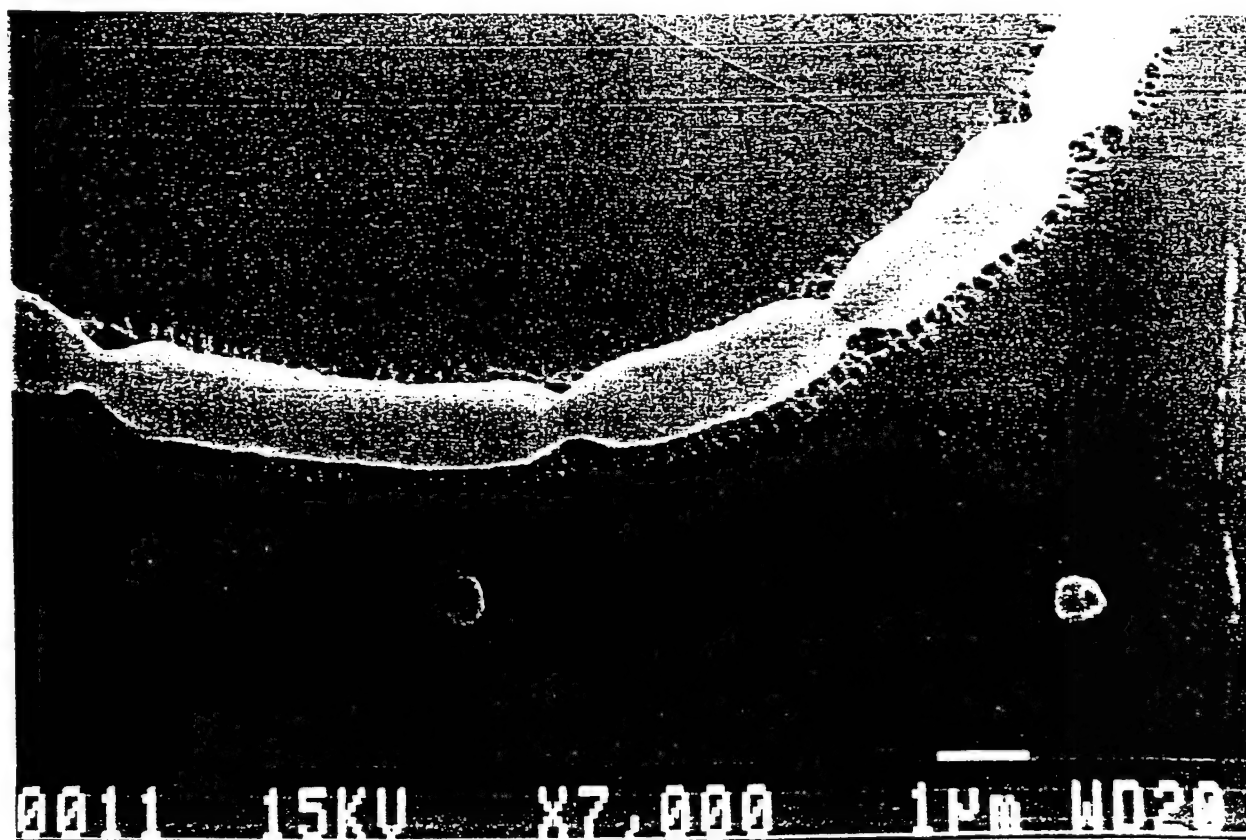


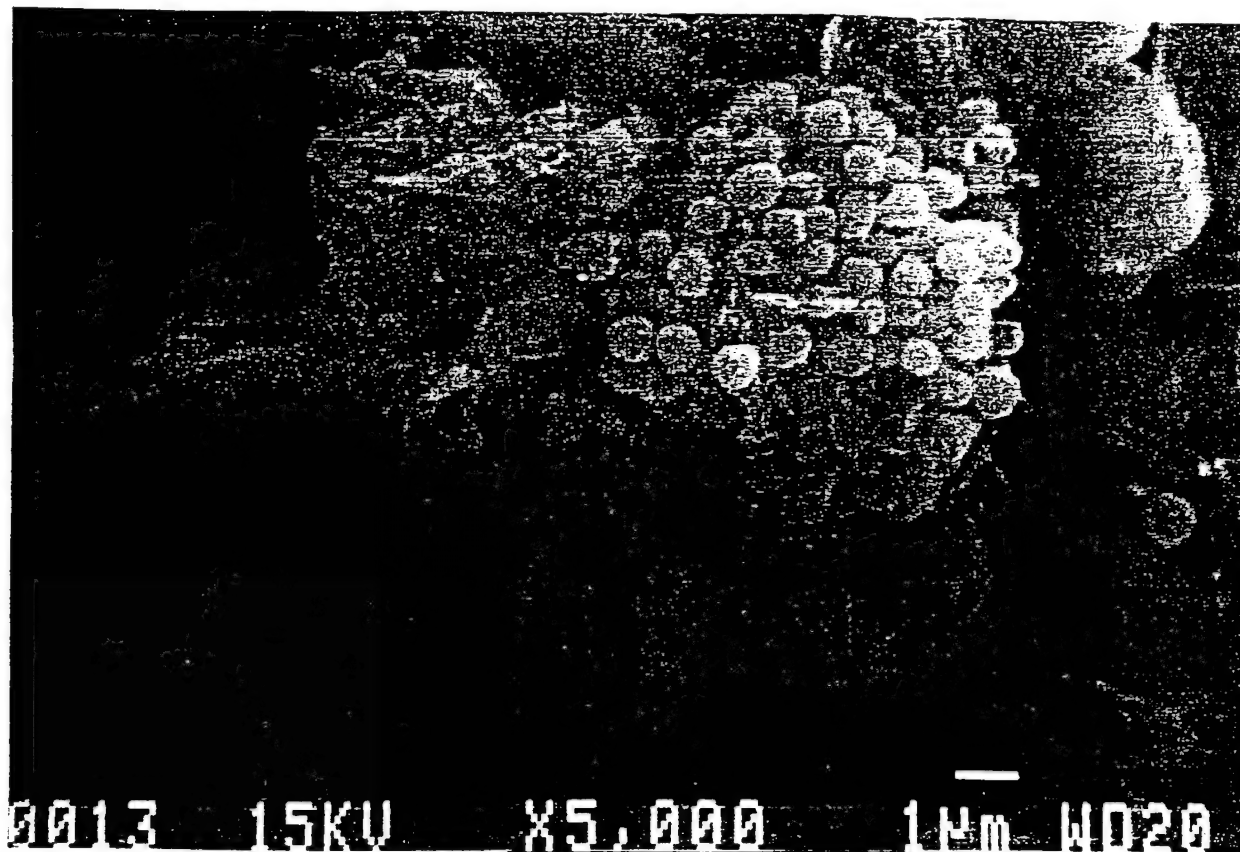
SEM for pond slides (brackish)



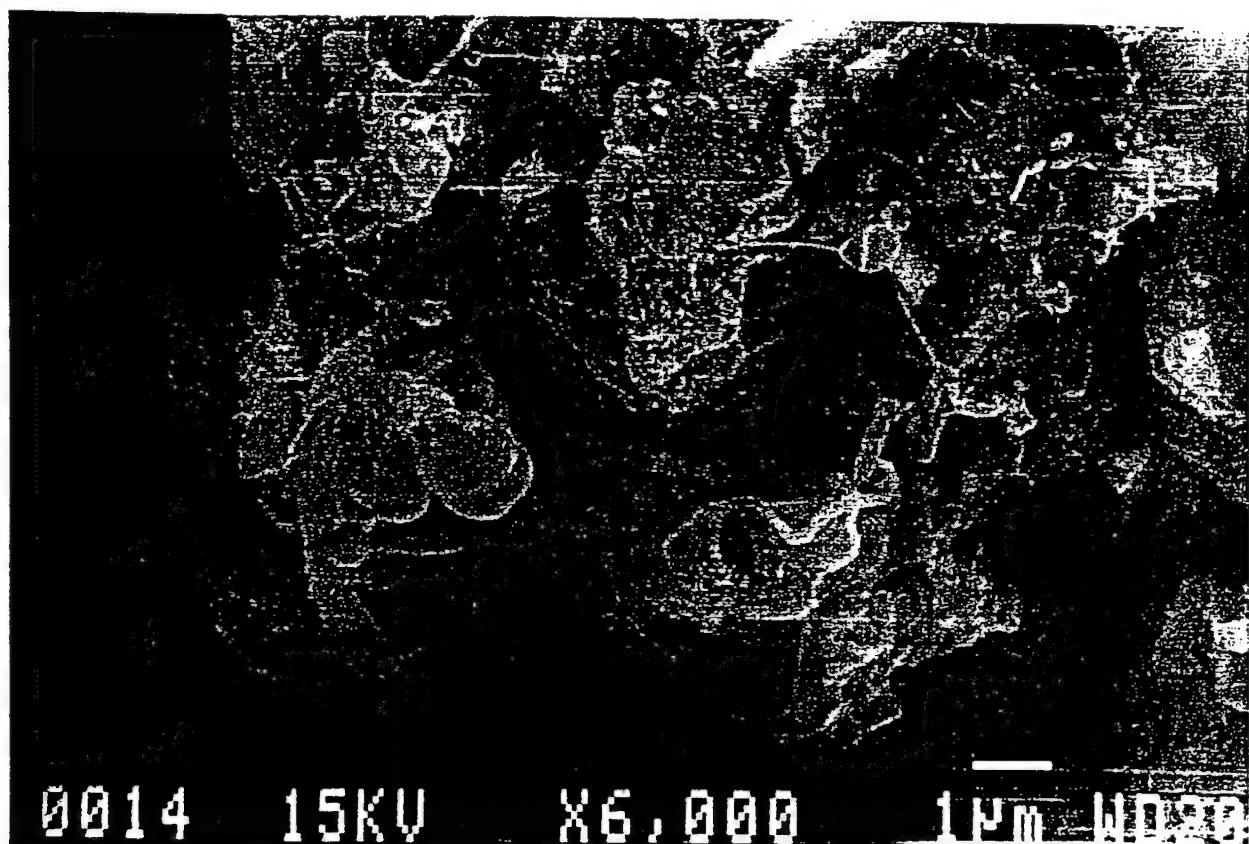


SEM for pond slides (brackish)



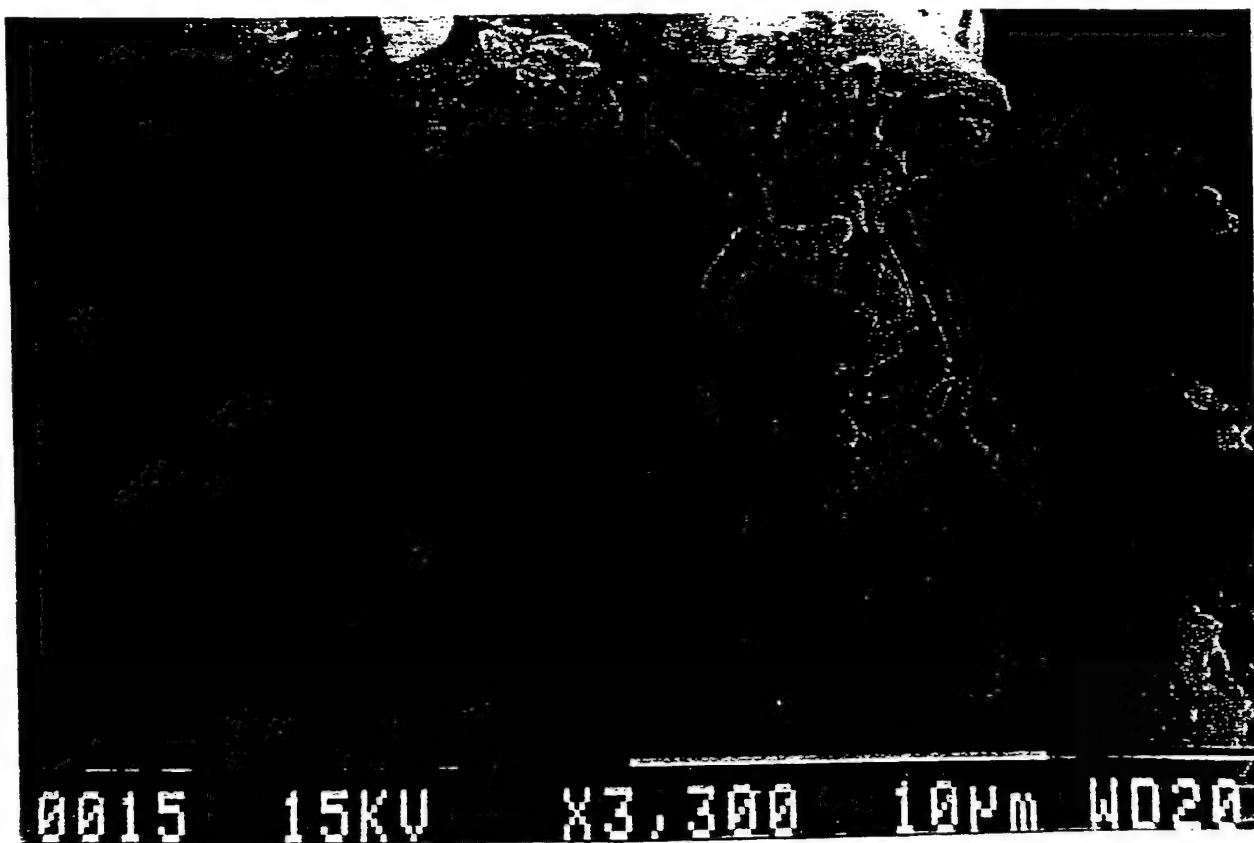


SEM for pond slides (freshwater)





SEM for pond slides (freshwater)



Enrichment of "Chlorochromatium aggregatum".

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## INTRODUCTION.

Among the green sulfur bacteria there are green and brown pigmented species. They can form different types of aggregates: star-shaped and net-shaped. Besides these aggregates there are also symbiotic associations described: *Chlorochromatium aggregatum* and *Pelochromatium roseum* (Schlegel, 1993).

"*Chlorochromatium aggregatum*" is a bacterial consortium which consists of two types of bacteria. One is an unidentified, slightly motile, sulfate reducing bacterium (SRB) and the other is a green sulfur bacterium. In the consortium the green sulfur bacterium is the ectosymbiont, loosely attached to the SRB. The metabolism of both organisms is linked, probably by the cycling of sulfate and sulfide: the sulfide produced by the SRB serves as an electron donor for the green sulfur bacterium which results in the formation of sulfate; the sulfate is then used as an electron acceptor by the SRB. There is not much known about the characteristics of this consortium. Earlier studies showed that the ectosymbiont disappears after some time and the enrichment will slowly degenerate and eventually dies. The enrichment of this consortium is a delicate work and nobody has ever succeeded in isolating this consortium.

The aim of this study is to develop procedures to enrich for the "*Chlorochromatium aggregatum*" consortium.

## MATERIALS AND METHODS.

### Sources of inoculum.

Samples were collected at different times and spots from Oyster Pond (Woods Hole): close to the inlet and further up the pond as well as from School Street Marsh (Woods Hole). In both cases the top 5-10 cm of the sediment were sampled and transported to the laboratory in closed erlenmeyers or sampling cores. Part of the samples were overlaid with water from the sampling site and the others consisted of plain sediment.

### Enrichments.

The first series of enrichments ( 7 erlenmeyers ) all consisted of sediments overlaid with sampling site water. The erlenmeyers were placed in front of a fluorescent lamp and incubated at room temperature without further additions. Each day, samples were taken for microscopy.

A second series of samples ( 6 samples were collected ) were taken from Oyster Pond. These enrichments were overlaid with prefiltered water from "Bell Tower Pond". After one day of incubation 10 mg/l of DCMU ( 3-(3,4 dichlorophenyl)-1,1-dimethylurea ) was added to inhibit the anoxygenic photosynthesis. Some dithionite was added

to reduce the environment. Microscopic examinations were done daily.

A third series of samples ( 6 samples were collected) were taken at Oyster Pond. Two samples were overlaid with prefiltered water from "Bell Tower Pond", two with water from the sampling site and two with a 1:1 mixture of prefiltered water from "Bell Tower Pond" and spring water from the cafeteria in Swope. Sodium sulfide (0.1 mM) was added to reduce the flasks and all were placed in front of the light set. Daily microscopical observations were done.

Enrichments were subcultured in modified PNSB media which contained the following (per liter):

0.34 g KH<sub>2</sub>PO<sub>4</sub>; 0.34 g NH<sub>4</sub>Cl; 0.34 g KCl; 0.5 g MgSO<sub>4</sub>; 0.3 g CaCl<sub>2</sub>. This solution was autoclaved and the following additions were made to finish the medium: NaHCO<sub>3</sub> 18 mM; Na<sub>2</sub>S.9H<sub>2</sub>O 2.5 mM; 6-vitamin solution 1 ml; Trace element solution SL 12 1 ml. Final pH of the medium was set at 7.0 with HCl.

In some cases the medium was used at a 20% concentration with some modifications: NaHCO<sub>3</sub> 15 mM; Na<sub>2</sub>S.9H<sub>2</sub>O 0.3 mM; Vitamin solution 0.5 ml and trace element solution 0.2 ml.

All incubations were done at room temperature.

### Microscopy.

Routine light microscopy was carried out with a Zeiss phase-contrast microscope.

Pictures were taken with a Zeiss axioscope microscope using phase contrast and polarized light. Flagella were examined using DIC microscopy.

### RESULTS AND DISCUSSION.

The first series of enrichments ( 7 erlenmeyers ) all contained a wide variety of microbiota like Beggiatoa ssp.; Chromatium ssp.; Thiovulum ssp.; several purple sulfur species; several green sulfur species and a lot of protozoa. But no "Chlorochromatium" could be observed. According to Overmann (personal communication) the microbial mat in Oyster Pond had a different look than previous years: there was a lot more activity of the "pink organisms at the top of the mat". This might explain the lack of "Chlorochromatium" in these enrichments. Also after 5-7 days no Chlorochromatium could be observed in one of the enrichments; neither from Oyster Pond, nor from School Street Marsh.

The second series of samples ( 6 erlenmeyers ) again contained a wide variety of microbiota, similar to the ones observed in the first series. A decrease of the amount of species could be observed during time, due to the addition of DCMU. After 7 days of incubation no Chlorochromatium was observed.

The third series of samples ( 6 erlenmeyers ) contained, again, a wide variety of microbiota in all enrichments. After 4 days Chlorochromatium could be observed in the enrichments with the 1:1 mixture of spring water and pond water. No Chlorochromatium could be observed in one of the other enrichments.

After 5 days, the amount of Chlorochromatium had increased even more. An explanation for the growth of Chlorochromatium in these enrichments might be that the amount of nutrients is too low for other bacteria to grow, and since Chlorochromatium requires a low nutrient level in order to grow.

Secondary enrichments were started by transferring 1 ml of the sediment surface into 30 ml sealed pyrex tubes with the modified PNSB medium at 100% at 20% strength. Parallel enrichments were started with 50 ml Pfennig bottles with 20% chromatium medium. When samples from the third series were examined using polarized light, the ectosymbionts could be observed quite clearly (See pictures in appendix). The amount of Chlorochromatium in comparison with the total amount of microbiota was estimated at 0.5-1%.

One remaining sample from the first series was also examined using polarized light. Also there some Chlorochromatium could be observed. The amount of Chlorochromatium in this sample was far less than 0.5% of the total population. The difference with the samples from the third series was, that the amount of ectosymbionts per consortium was around twice as much (See pictures). The consortia in this enrichment were already incubated for almost 3 weeks. This means that in this type of enrichment (sediment overlaid with sampling site water) the Chlorochromatium could be maintained, and slowly enriched.

An attempt was done to show the flagella of the consortium using DIC microscopy. The results were not so clear, because the consortium was floating through the sample all the time. The picture shows in two of the four pictures a vague curved flagellum. From the microscopical examinations one could see that the flagella were not attached to the ectosymbiont, but came from the central organism at the lateral side.

## CONCLUSIONS.

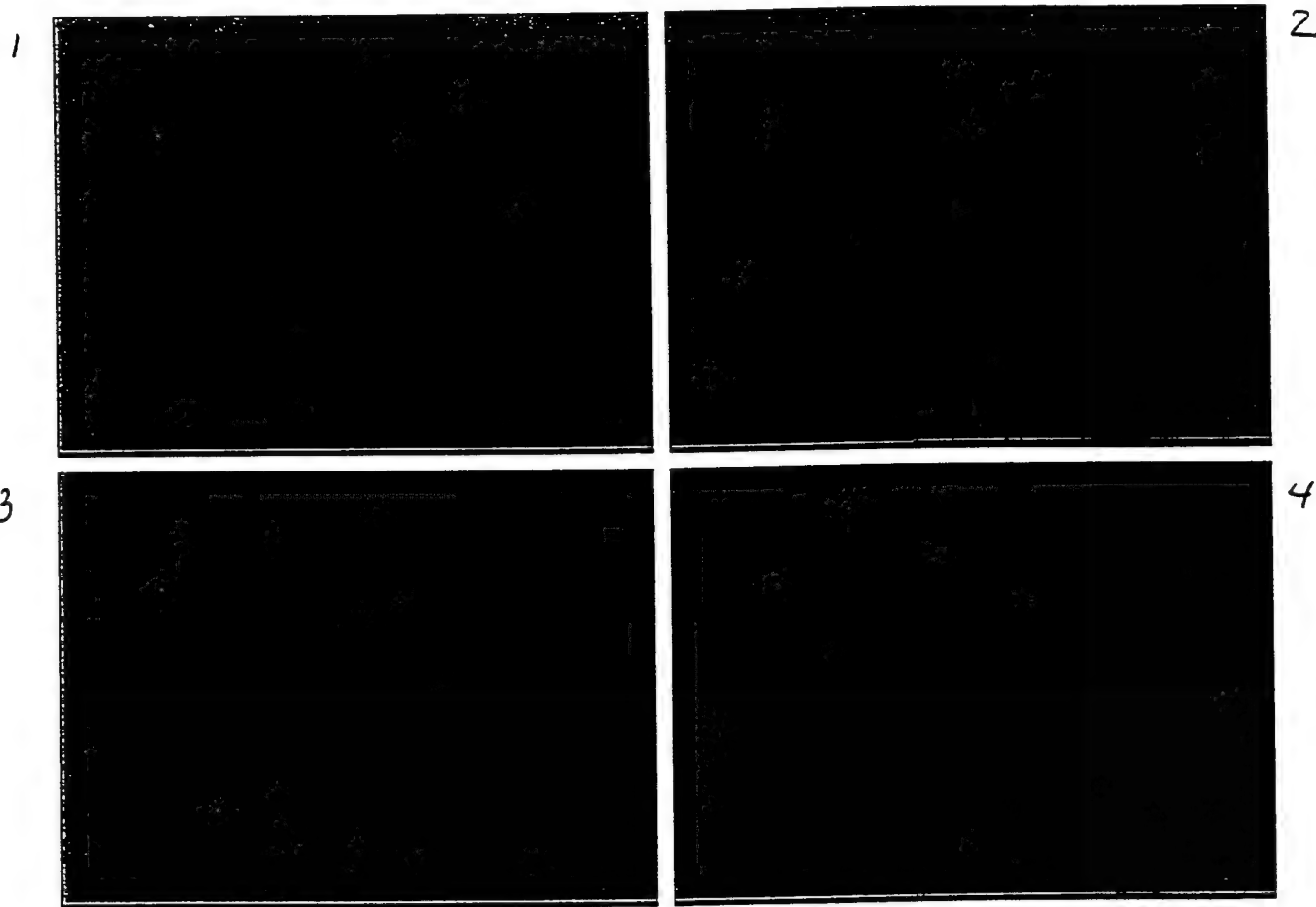
- "Chlorochromatium aggregatum" can be enriched from Oyster Pond, using plain sediment overlaid with sampling site water or Bell tower pond water mixed (1:1) with cafeteria spring water.
- The amount of ectosymbionts per central cells in older enrichments increases.
- The flagellum of the consortium is probably attached at the central cell.

#### LITERATURE.

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Appendix



Polarized light picture of Chlorochromatium aggregatum  
from third series samples (1,2,3) and first series (4)

more pictures available in course files.

Thanks Tom!!!! 

**Degradation of methanesulfonate by aerobic bacteria.**

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## INTRODUCTION.

In the marine organic sulfur cycle Dimethylsulfide (DMS) plays a significant role: over 30% of the total volatile sulfur compounds are due to the production of it. The DMS is derived from the degradation of dimethylsulfoniumpropionate (DMSP), which is produced by marine algae, cyanobacteria and marshplants Kelly et al., 1990) A recent study by Kiene and Hines shows that *Sphagnum* sp. in peatlands are also a considerable source of DMS.. The occurrence of methanesulfonate (MSA) in the atmosphere is due to the photochemical destruction of DMS. MSA is a stable compound and a strong acid. It does not undergo chemical breakdown in the atmosphere, but is deposited on the Earth's surface in rain and/or snow. There is little known in the literature about the biodegradation of MSA. It can be used as a sulfur source by p.e. *Chlorella fusca* as well as by some bacteria isolated from soil, sewage and certain enteric bacteria. MSA can be used as a sole source of carbon and energy by a novel Gram-negative methylotrophic bacterium, isolated from the soil. There is a recent report describing two marine strains which can degrade MSA in a similar way. There is, to my knowledge, no report of bacteria which are able to use MSA the sole carbon, energy and sulfur source.

The aim of this study was to obtain more information about the degradation of MSA under aerobic conditions.

## MATERIALS AND METHODS.

### Media.

For batch cultures a mineral salts medium was used with the following composition per liter of deionized water: 1.2 g  $K_2HPO_4$ ; 0.6 g  $KH_2PO_4$ ; 0.05 g  $CaCl_2 \cdot 2H_2O$ ; 0.16 g  $MgCl_2 \cdot 6H_2O$ ; 0.5 g  $NH_4Cl$  and 2 ml of trace element solution SL12. In some cases 0.02% yeast extract was added.

Phosphates were sterilized separately. The final pH was set at 7.2 with HCl. Methanesulfonate (MSA) and methanol (MeOH) were filter sterilized in stock solutions of 1 M and added to the sterilized media. If necessary, a S-source was added as  $Na_2SO_4$ .

In some cases 0.02% yeast extract was added together with the "six vitamin solution" and vitamin B12.

When using agarplates, the MSA medium was supplied with 2% washed agar (washed twice) or nutrient agar.

Media for the chemostat had the same composition as described above, except that the  $MgCl_2$  and the  $CaCl_2$  were sterilized separately and added to the sterilized media.

#### Type of inoculum.

Different sources of inoculum were used: soil from the garden near the "Brick Dorm"; sediment from Cedar Swamp and sediment from School Street Marsh. The chemostat was inoculated with a mix of these inocula ("Supermix").

#### Enrichment procedures.

Enrichments were started in liquid media and on agarplates with MSA as the sole carbon, energy and S-source as well as in liquid media with additional carbon and S-sources. All incubations were done at 30 C

Chemostat enrichments were started by inoculating a 1 l vessel, at a dilution rate of 0.1 per hour. The vessel was equipped with pH and temperature control. A constant temperature of 30 C and a pH of 7.1 were set throughout the experiment and air was supplied to the vessel (constant, but uncontrolled).

Growth in the chemostat was checked daily by measuring the OD 660nm and by microscopy. Samples were plated on MSA agar with MSA or MeOH as the carbon source as well on nutrient agar plates.

#### Analytical methods.

MSA and sulfate were measured by HPLC (Waters), equipped with an anionexchange (IC-PAK A HC) column eluted with gluconate/borate. The eluens was prepared as follows:

1 liter solution A contained: 16 g sodium gluconate, 18 g boric acid, 25 g sodium tetraborate decahydrate (Borax) and 250 ml glycerol. One liter of solution B (eluens) contained: 20 ml sole. A, 20 ml. 1-butanol and 120 ml acetonitrile. The eluens was filtered through a 0.22 micron filter and degassed before use. The flow rate of the HPLC was 2 ml/min. The detector was a 430 Waters conductivity meter running at 35 degrees.

#### RESULTS.

##### Chemostat enrichments.

After two days of incubation, some turbidity could be observed in the chemostat. The turbidity increased further in time (Table 1).

Table 1: Optical density, pH and MSA concentration in the chemostat.

Time (days)	OD 660 nm	pH	MSA mM
0	0.01	7.2	nd.
1	0.01	7.1	15.5
2	0.022	7.1	15.5
3	0.022	7.1	14.2
4	0.04	7.1	13.1
5	0.045	7.1	nd.
6	0.05	7.1	9.8
7	0.051	7.2	7.5
8	0.05	7.2	7.2

The chemostat had to be stopped after 8 days.

All samples taken from the chemostat showed growth on the three types of plates used. On MSA agar a shift in the colony types was observed in time: the first two days *Streptomyces* type cells were present besides short fat rods, but further in time the short fat rods were the only morphological types which could be observed on the MSA agar plates. On MeOH agar plates, similar colony types were found. On nutrient agar a wide variety of morphotypes could grow. None of the colonies growing on MSA agar plates were able to grow in liquid culture, even after the addition of 0.02% yeast extract, Na<sub>2</sub>SO<sub>4</sub> and vitamins to the medium. However the colonies could grow in nutrient broth and medium with methanol.

Two pure cultures (msacp1 and msacp2) were isolated from the liquid cultures. Both strains were Gram negative, short fat rods. Msacp1 was motile, oxidase positive and katalase negative. Msacp2 was slightly motile on nutrient broth, but showed no motility on other media, and was slightly oxidase positive and katalase negative. Both strains (msacp1 and msacp2) could only grow under aerobic conditions. Growth was observed after 4 days of incubation with methanol, methylamine and formate as the substrate. No growth was observed after 4 days of incubation on MSA, methane, glucose, acetate and taurine. No growth was observed under anaerobic conditions (in the presence of nitrate) on methanol, methylamine, formate, MSA and methane.

#### Liquid culture enrichments.

In all liquid cultures some growth occurred, after two weeks of incubation, but this was not always correlated to substrate degradation. Growth was measured as optical density (table 2).

Table 2: Optical density of enrichments in liquid cultures with different carbon and sulfur sources after 2 weeks of incubation.

C and or S source	OD 660 nm.
10 msa	0.05
20 msa	0.05
msa + meoh	0.15
msa + sulfate	0.05
msa + meoh + sulfate	0.25
10 meoh	0.15
20 meoh	0.2
meoh + sulfate	0.25
blank without substrate	0.05

Samples from all cultures, except the blank, were taken and secondary enrichments were started on MSA, MeOH and nutrient agar. In all cases a wide variety of colony types grew on the nutrient agar. A few colony types grew on the MeOH agar and no growth was observed on the MSA agar plates, except for the MSA/MeOH enrichment. Several colonies from the methanol plates were restreaked and were obtained in pure culture. Three different morphological types could be distinguished: a pale white colony-forming short motile rod; a shiny white colony-forming fat motile rod and an irregular pink colony forming non-motile cocci. All were Gram-negative and oxidase positive. The colonies from the MSA agar were inoculated into liquid medium, but showed no growth after one week.

## DISCUSSION.

### Chemostat enrichments.

The organisms enriched in the chemostat were able to convert MSA (see table 1). Within one week the amount of MSA decreased from 15.5 mM to 9.8 mM. Since no additional carbon and/or sulfur sources were added to the medium, this degradation was done by microorganisms, which are able to use MSA as the sole carbon, energy and sulfur source.

The secondary enrichments on plates showed some growth, but after transferring them into liquid medium with MSA as the sole carbon, energy and sulfur source no growth was observed within one week. There are some speculations to make about this phenomena. It could be that the bacteria which could grow on the MSA agar plates were growing on traces of carbon in the agar, but couldn't use MSA as a substrate. Another speculation might be that MSA was degraded by two organisms, where one converts the MSA into an intermediate plus sulfate and the other converts the intermediate into CO<sub>2</sub>. Possible intermediates are methanol, formate or formaldehyde.

Reactions which could be involved in the conversion of MSA:

- 1a)  $\text{MSA} + 2 \text{ O}_2$  ----->  $\text{CO}_2 + \text{H}_2\text{O} + \text{H}_2\text{SO}_4$
- 1b)  $\text{MSA} + \text{O}_2$  -----> formaldehyde/methanol/formate +  $\text{H}_2\text{SO}_4$
- 2)  $\text{methanol} + 1.5 \text{ O}_2$  ----->  $\text{CO}_2 + 2 \text{ H}_2\text{O}$
- 3)  $\text{formaldehyde} + \text{O}_2$  ----->  $\text{CO}_2 + \text{H}_2\text{O}$
- 4)  $\text{formate} + 0.5 \text{ O}_2$  ----->  $\text{CO}_2 + \text{H}_2\text{O}$

It might be that the second organism could grow faster and was isolated instead of the MSA degrader, which might not grow on agar or very poorly. Thompson et al. also mention that the MSA degrading bacteria TR-3 and PSCH4 that they isolated were only able to grow on less than 0.5% agar noble. Since we used 2% normal agar this may be an explanation for the lack of growth of our MSA degrader. The inability of methylotrophs to grow on agar (Lees et al; 1991) seems to be relatively common. In commercial preparations of agar there may low concentrations of inhibitory compounds.

The two isolated strains (msacp1 and msacp2) are methylotrophic strains, since they can grow on all the C-1 compounds tested (except for MSA). It is not sure whether they are obligate methylotrophs, since only a few other carbon sources were tested for growth. They are not responsible for the MSA degradation in the chemostat, but probably could grow on an intermediate from the MSA degradation.

#### Liquid culture enrichments.

The enrichments in liquid cultures showed that methanol was degraded very easily and therefore that it is easy to enrich for methylotrophs from soil or sediment.

But after 2-3 weeks of incubation no growth could be observed in the enrichments with MSA. So it can be concluded that within the time of the experiments MSA is not degraded in batch cultures, and none of the other enrichments was able to convert MSA in secondary enrichments.

#### CONCLUSIONS.

-A chemostat is a useful tool for the enrichment of MSA degrading micro-organisms.

-Methanol is an easily degradable substrate for methylotrophs and it is relatively easy to obtain isolates from these enrichments.

-Liquid culture enrichments maybe are useful tools to enrich for MSA degrading organisms, but not a longer incubation time is needed for them to develop.

-Several pure cultures could be isolated during the time period of 3.5 weeks.

## RECOMMENDATIONS

for future students/research

-It would be useful to have a chemostat running during the whole period of the project, especially because the doubling time of the MSA degrading organisms is around 10 hours. It takes a while to get a steady state in the reaction vessel and to obtain enough biomass to do further enrichments.

-Several agartypes should be tried out in order to get the MSA degrading bacteria growing (agar noble, phytigel plates, lower concentrations of agar).

## LITERATURE.

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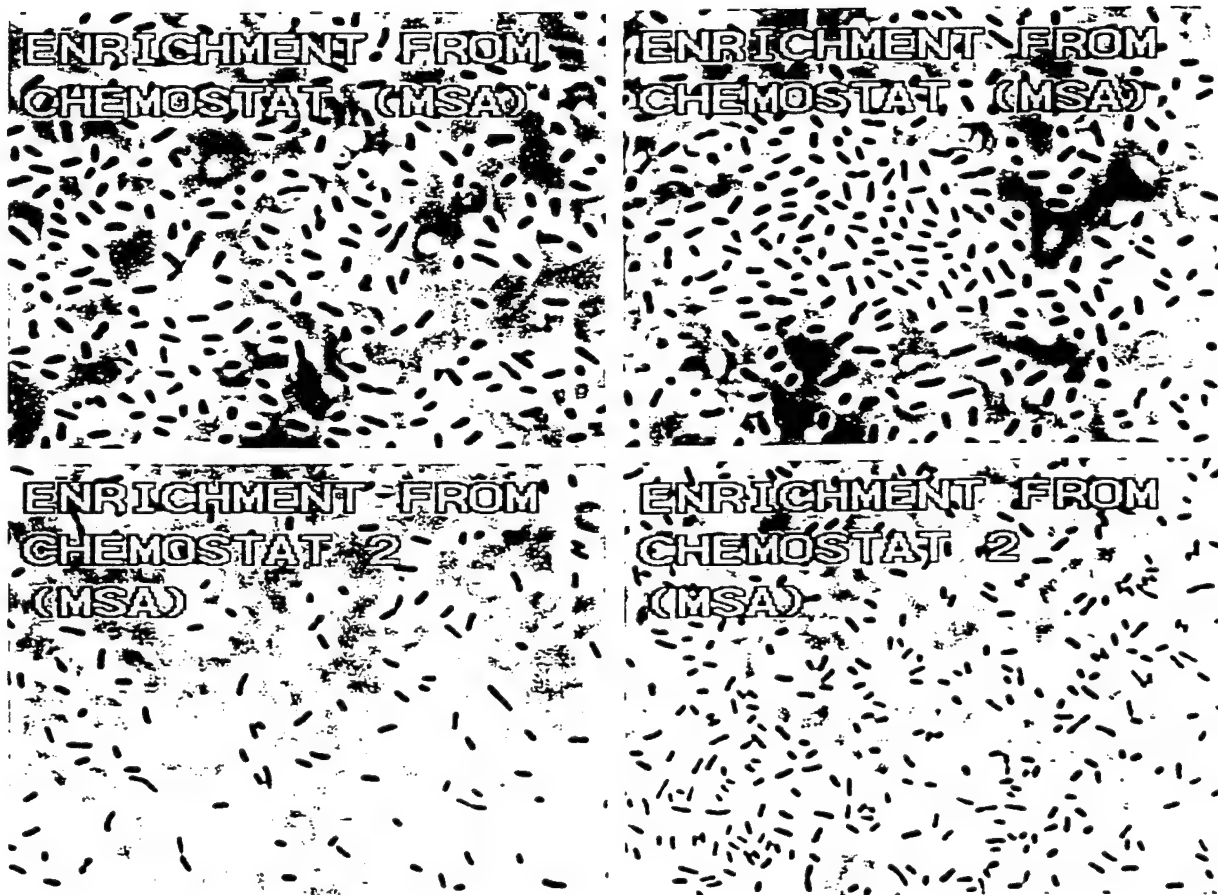
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# Appendix



Photographs (phase contrast) of  
secondary enrichments from the chemostat  
grown on methanol-agar plates

Use of Canada Balsam as a Selective Agent and Possible Substrate in the  
Enrichment of Termite Hindgut Microbiota

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#### ABSTRACT

The termite gut microbiota naturally encounter high levels of resin compounds. I show that they are resistant to high levels of resin in culture, and that this resistance can be used as a selective technique for termite hindgut anaerobes. Suspended resin compounds are precipitated, solubilized, chemically degraded or metabolized in the course of culturing hindgut organisms. An attempt was made to determine whether metabolism was, in fact, occurring; results were inconclusive.

## INTRODUCTION

The intestinal tracts of most animals include oxygen-poor and anoxic environments suitable for the growth of anaerobes. Beyond this basic similarity, however, the gut environment varies: different animal species have distinctive diets which in turn provide a distinctive, species-specific gut environment.

This specificity has been used to enhance the success of culture techniques; for example, rumen fluid is used to culture the rumen microbiota of cattle (reference). For other animal gut cultures, this approach, while feasible, is less practical. In these cases, introducing compounds characteristic of the host diet in the culture medium may increase both specificity and yield of gut microbes. To do so, a compound should have the following characteristics: First, it should be something that the anoxic gut microbiota would actually encounter; that is, it should not be rapidly metabolized by the host early in the digestive process. Next, the compound should be characteristic of the standard diet, so that the microbiota are likely to be well-adapted to its presence. Ideally, active selection might be possible. In any diet, there are generally compounds present that have some level of anti-microbial activity; if these can be detected and employed, they might encourage growth of the "true," relatively resistant gut microbiota by concurrently inhibiting growth of sensitive adventitious organisms. Finally, added compounds might serve as a nutrient source for the gut microbiota. It should be noted that while this is the most obvious role for a medium-additive, it is not actually a necessary one; culture success depends on selectivity as much as upon adequate nutrition.

I have investigated the use of mixed resin compounds (Canada Balsam) in the culturing of obligate and facultative chemolithotrophs from the termite hind-gut. The termite diet is distinctive for the range of noxious resin compounds encountered; this, along with the exceptionally high concentration of cellulose, would serve to define the diet. Therefore, it is reasonable to expect that the gut microbiota survive in the presence of resin compounds. It is also possible that resin, which contains both volatile aromatics and larger, multicyclic organic compounds, serves as an auxiliary carbon source. A variety of acetogenic bacteria and a few methanogenic archaea are facultative chemolithotrophs, and are known to metabolize such simple compounds as sugars, alcohols, organic acids and amines (reviewed in Gottschalk, 1992, see also Schink 1994); among known termite-gut isolates, one, *Acetonea longum*, falls into this group.

Because resin derivatives have a wide range of uses not yet subsumed by petrochemical equivalents, metabolism of resins is potentially of wide interest. In art conservation, controlled removal of photochemically darkened resins and waxes might be useful; by contrast, conservation of old wooden structures requires that pine tars and pitch be preserved.

## METHODS

### Enrichments

The hindgut of two termites (Woods Hole specimens, species undetermined) were dissected in a drop of AC21 reduced medium (Breznak reference). A methanogen-enriching primary culture was prepared using AC21 anoxic culture tubes (3 ml liquid medium under  $H_2/CO_2$  gas phase at a pressure of approx. two atmospheres, incubated at 30°).

Upon evolution of methane, the culture was sub-cultured at high concentration (1:10 dilution) on AC21 bottle plates containing increasing concentrations of Canada balsam (0, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0 and 3.0 % weight/volume Canada balsam). These plates were prepared by adding the appropriate volume of a 10% solution of Balsam in EtOH, then purging the EtOH (and balsam volatile compounds) by repeated gas exchange over the cooling agar medium, initially with moderate shaking to promote boil-off (at approx. 75-60°).

After colony formation, excess liquid medium from the base of the bottle plates (turbid run-off from plating) was used to inoculate further AC21 liquid cultures, in a limiting dilution series ( $10^{-2}$  to  $10^{-10}$ ). At most dilution levels, tubes were set up containing either .1% Canada balsam in EtOH, EtOH alone, or no additions. Finally, roll tubes were inoculated either by streaking or by addition of liquid culture from the (supposedly) limiting dilutions.

For bottle plates, CB was autoclaved with the medium either once or twice; for roll tubes, it was added prior to melting of the agar by boiling; and for liquid cultures, it was added without further heating. Heat-based hydrolysis of resin compounds could play a roll in rendering them metabolizable. In the future, all CB-containing media should be autoclaved after aliquotting and addition of CB.

### Metabolic analysis

Evolution of methane was detected by gas chromatography (GC). Methanogens were also detected by characteristic  $A_{420}$  fluorescence. Potential metabolism of Canada balsam was quantified by extraction of both pellet and supernatant fractions (2.0 ml) with 1 or 2 ml methylene chloride (respectively). After vacuum evaporation of methylene chloride, constituents were re-suspended in the appropriate HPLC solvent phase and analyzed on a C18 column. As a variant on the ramped HPLC method of Kutney et al. (1988), two non-ramped HPLC runs were performed to elute different resin acid compounds. The two solvents used were 87% and 70% methanol containing .1% acetic acid as carrier, at a flow rate of 1.5 ml/min.

## Hybridization

To predict possible drug resistance among the bacteria (a potentially useful aid to further directed purification of the enrichments), colony lifts were taken from one of the bottle plates and colonies were probed for Tet Q and Tet M resistance genes. Filter preparation was essentially as by standard methods (e.g. Sambrook et al.) with extended denaturation and (for one filter) autoclave lysis (1 min). Probe hybridization and detection were as described in the Dupont NEN Renaissance Kit <sup>(TM)</sup>.

## Gram Stain

Staining was done following the directions in the stain kit.

## RESULTS

### Canada Balsam is Tolerated

As hoped for, the termite gut contains microorganisms that are unusually resistant to high levels of Canada Balsam (CB). In fact, growth was observed on bottle plates up to a level of .3% CB. In comparison, when resin acids are used as a growth substrate or nutritional "challenge" for bacteria and fungi that metabolize them aerobically, the concentration used is in the range of .003-.004%. Because CB contains a range of acidic and non-acidic resins, it is unwise to assume that toxicity is equivalent. However, the resin acids are a considerable proportion of the ethanol-soluble fraction of CB (certainly more than one part in a hundred, and probably more than one part in ten). Anecdotaly, it should be noted that observation of termite gut contents shows fluorescent globules that resemble the globules of CB seen under these culture conditions, further supporting the assertion that CB is a natural constituent of the termite hind-gut contents.

### Canada Balsam Enhances Growth

The growth of methanogens and bacteria on the bottle plate with no CB was feeble. Eventually (> 10 days) a lawn of micro-colonies began to appear. By contrast, growth of individual colonies was vigorous on/in bottle plates containing .003 and .03% CB. It was more difficult to assess relative growth rates in liquid culture because CB forms a milky suspension in the liquid medium. CB also can interfere with microscopic analysis because the suspension includes many droplets that are in the size-range of bacteria. As mentioned above, these also fluoresce during  $A_{420}$  detection of methanogens. After several days (10+), growth was clearly no worse in CB tubes than in tubes without CB.

### Canada Balsam is Selective

Upon observation of the (relatively) vigorous growth on/in CB-containing bottle plates, distinct colors and morphologies could be seen. When picked for microscopic analysis, the rare, larger, opaque cream-colored colonies were found to correspond to a methanogen (Fig. 1a). Additionally, the major methanogen seen (by  $A_{420}$  fluorescence) in the primary culture was enriched in the fluid "run-off" at the bottom of the .1% CB bottle plate, compared to the balsam-less plate (Fig. 1b). However, this selectivity has its limits, at least with respect to the methanogens. Methanogen-like colonies made up considerably less than 1% of the colonies on any of the plates. As expected from this number, methanogens were not brought into pure culture by a limiting dilution strategy (Fig. 1C, and dilutions beyond  $10^{-8}$ --not shown).

### Canada Balsam is Altered

Tubes inoculated with a dense culture of bacteria showed a clearing of the CB suspension within three days. Tubes inoculated with a more dilute culture "cleared" after longer incubation. Control tubes (no cells) never "cleared." At the same time, flocculent particles appear, and drop out of solution as a loose precipitate. One possibility is that the CB is somehow being metabolized (see discussion). To determine if metabolism was happening, HPLC analysis of the culture was carried out. Three vials from the dilution series were used. The  $10^{-6}$  dilutions were chosen because growth had been vigorous but not instantaneous (assuring that effective selection could have taken place). These vials showed a range of cell types by microscopic examination (Fig. 1c) as did roll tube cultures derived from them (Fig. 1d).

Unfortunately, GC analysis was impeded by the finding that the gas phase over a supposedly pure CB and ethanol mixture was evolving a peak overlapping or corresponding to methane (Fig. 2a). The shift in the relative size of methane and other peaks, therefore, should be treated with caution. HPLC analysis was impeded by the too-small sample size. For both pellet and supernatant fractions, the CB-specific peaks were hard to detect relative to the baseline. One HPLC run was intended to show any drop in slower-eluting substrates; the other, to show any rise in the (generally) faster-eluting metabolites of these substrates. No reproducible change was seen for substrates (data not shown). The results for metabolites were only slightly more suggestive, though again signal-to-noise ratio was very low. However, there was a suggestion of a new or amplified peak (Fig. 2b). The composition of this peak remains unknown.

### Gram Staining and Colony Hybridization can be Done

In the absence of positive controls, staining was inconclusive (Fig 1d). One set of spore-forming long rods may be positive, though the spores themselves are

not. Colony hybridization was also negative. It was unclear whether this was due to the choice of probes, or to the fixation of adequate DNA on the filter. At a minimum, we now know that it is possible to lift colonies cleanly from a bottle plate. (It should be pointed out that this destroys the plate--at least, no re-growth was seen after 10 days additional incubation.)

## DISCUSSION

Canada Balsam may turn out to be a useful selective agent. For pure culture of methanogens, it should probably be combined with a more standard antibiotic selection scheme (to enhance the proportion of archaea). A variety of spore formers seem to survive CB and ethanol selection. It is unlikely that they thrive both in the presence and absence of CB--if they were metabolizing efficiently, they presumably would not sporulate in large numbers. Because many of the (putative) spores were seen following a mixed culturing strategy (.1% CB plate run-off----> .1% CB liquid culture---->non-CB roll tube), it is hard to say where proliferation occurred and where sporulation occurred.

The whole question of Canada Balsam utilization is difficult to approach. For example, it would be unprecedented for methanogens to metabolize multi-carbon cyclic compounds. Yet, on CB plates, methanogens colonies grow reasonably well. In addition, the evolution of methane from the bottle plates is essentially undetectable, in spite of the presence of "methanogens." This could mean either that the colonies are not numerous enough to produce much methane, or that the methanogens have an alternate metabolism that they employ while growing on CB. Because all platings have been quite dense, it is (remotely) possible that diffusion from nearby colonies of bacteria could provide metabolically-limited methanogens with bacterially-produced CB metabolites. Due to the presence of a large volatile peak in the less-sparged ethanol-CB liquid cultures, evolution of methane from those cultures could not be meaningfully assessed.

HPLC analysis is both sensitive and flexible enough to approach questions of this nature; however, the run conditions must be optimized, and the size of the cultures used for analysis should be scaled up to at least 50 ml each to bring the signal associated with any one compound up above the general drift of the machinery and above the levels of generalized contaminants. Also, as a technical tip, it is important to flush the HPLC injector with the soluble phase at least 4 or 5 times between runs, as resin compounds stick avidly to a variety of surfaces (including glass).

Another interesting possibility is that resin compounds optimize some condition not directly related to metabolism. For example, they could improve adhesion to the agar surface, and also provide a non-aqueous substrate for growth in liquid culture. As a counter-argument, however, droplets of CB in liquid culture were

not associated with attached microbial growth. Alternately, the effect on the microbiota and on the balsam could have been affected indirectly, perhaps by mutual contributions to a change in pH.

Finally, it is possible that CB supports microaerophilic growth, and that this growth helps to return the enrichments to truly anoxic conditions optimal for growth of methanogens and other obligate anaerobes--in other words, the effect is biologically mediated by transient growth of organisms that do not grow to (or remain at) high density, and would therefore not be observed at advanced time points.

## REFERENCES

## FIGURES

### Figure 1

a) differential growth is already seen on the first set of bottle plates. methanogens are present primarily in the CB-containing plates (e.g. arrowhead, bottom left and bottom right), while filamentous chains are forming in the CB-less plate. This may correspond to the first step in spore formation seen in later cultures. Blue = fluorescence characteristic of methanogens; green = CB autofluorescence (as also seen in termite guts).

b) example of a methanogen colony "pick" from the .1% bottle plate--again, note blue fluorescence. Also note the internal structure (gas? inclusion?).

c) Serial dilutions aid selectivity of the enrichments. "-N" = power of the dilution; matched dilutions contain AC21 alone, AC21 + 1% EtOH, or AC21 + .1% CB and 1% EtOH.

d) Spore formers predominate following passage from CB to non-CB media; this also suggests possible selectivity in action, and growth followed by nutrient limitation.

### Figure 2

a) In GC, methane is possibly evolved as a peak that is also present in CB directly; these are the main peaks present (not identified).

b) HPLC analysis shows a possible increase in an unidentified peak of the cells + CB + EtOH sample that is not present in cells alone or cells + EtOH. However, a similar peak is present when a dense culture grown in the absence of CB has CB added immediately prior to sample preparation. Therefore, if this is a degradation product, the degradation must happen quickly and (presumably) via an extracellular process.

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Mohn, W. 1995. Bacteria Obtained from a Sequencing Batch Reactor that are Capable of Growth on Dehydroabietic Acid. *Applied and Environmental Microbiology* 61: 2145-2150

All Other References are to course handouts and xeroxes, with general information taken from The Prokaryotes.



1a

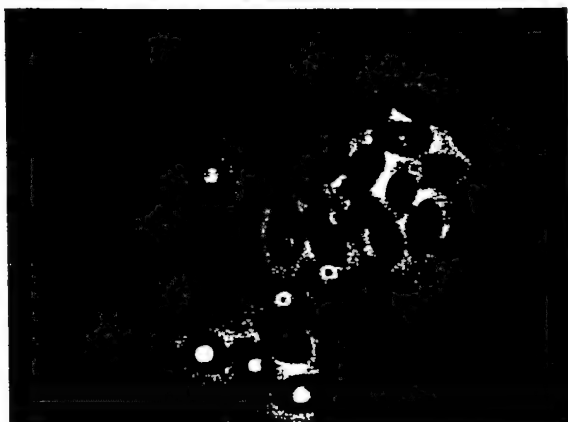
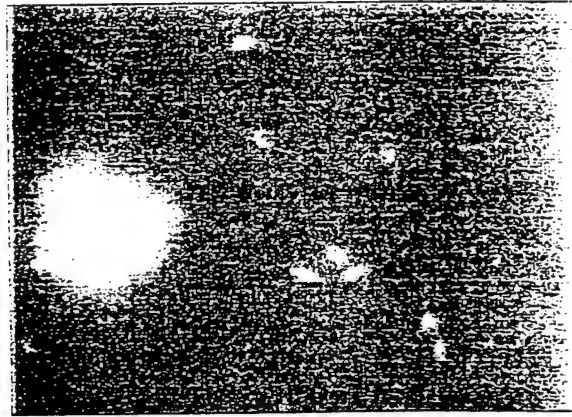
bottle plate  
"Ø"  
no canada  
balsam



bottle plate  
.1 %  
canada  
balsam

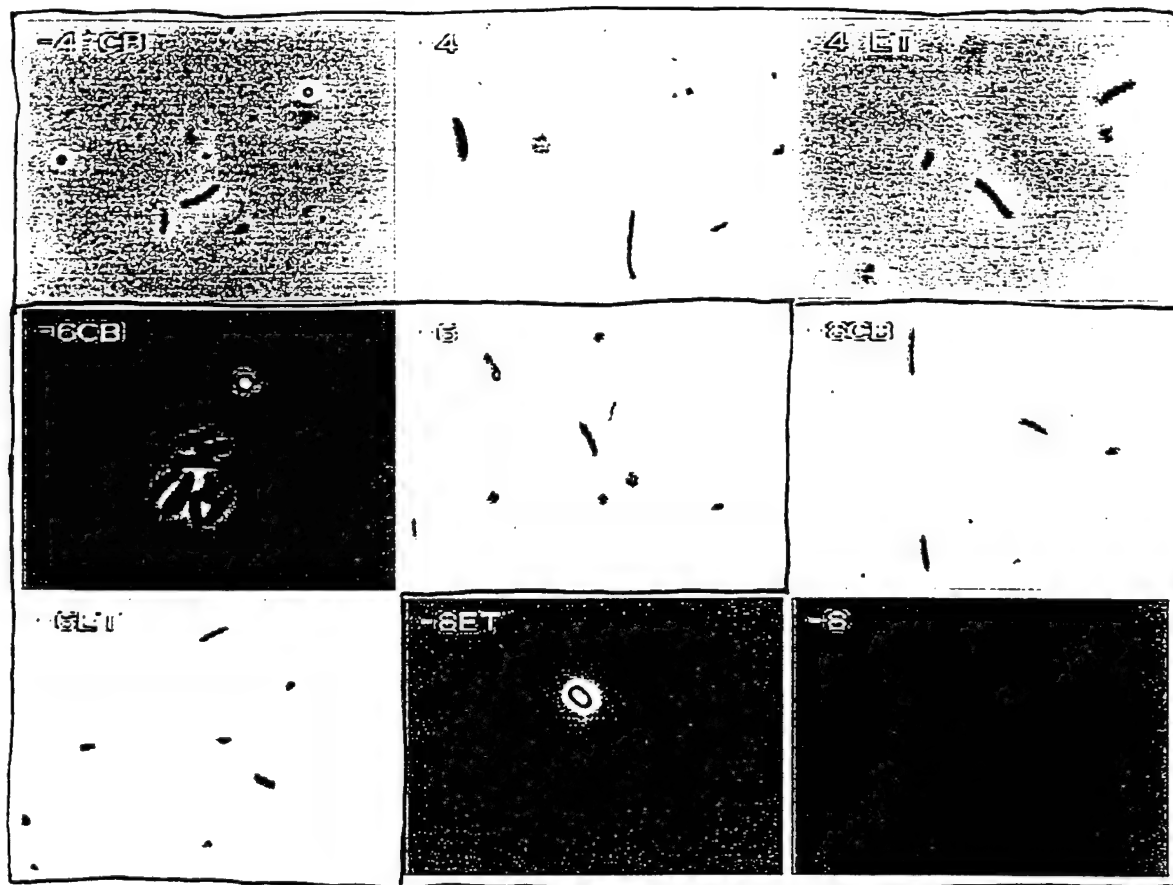


1b



JS

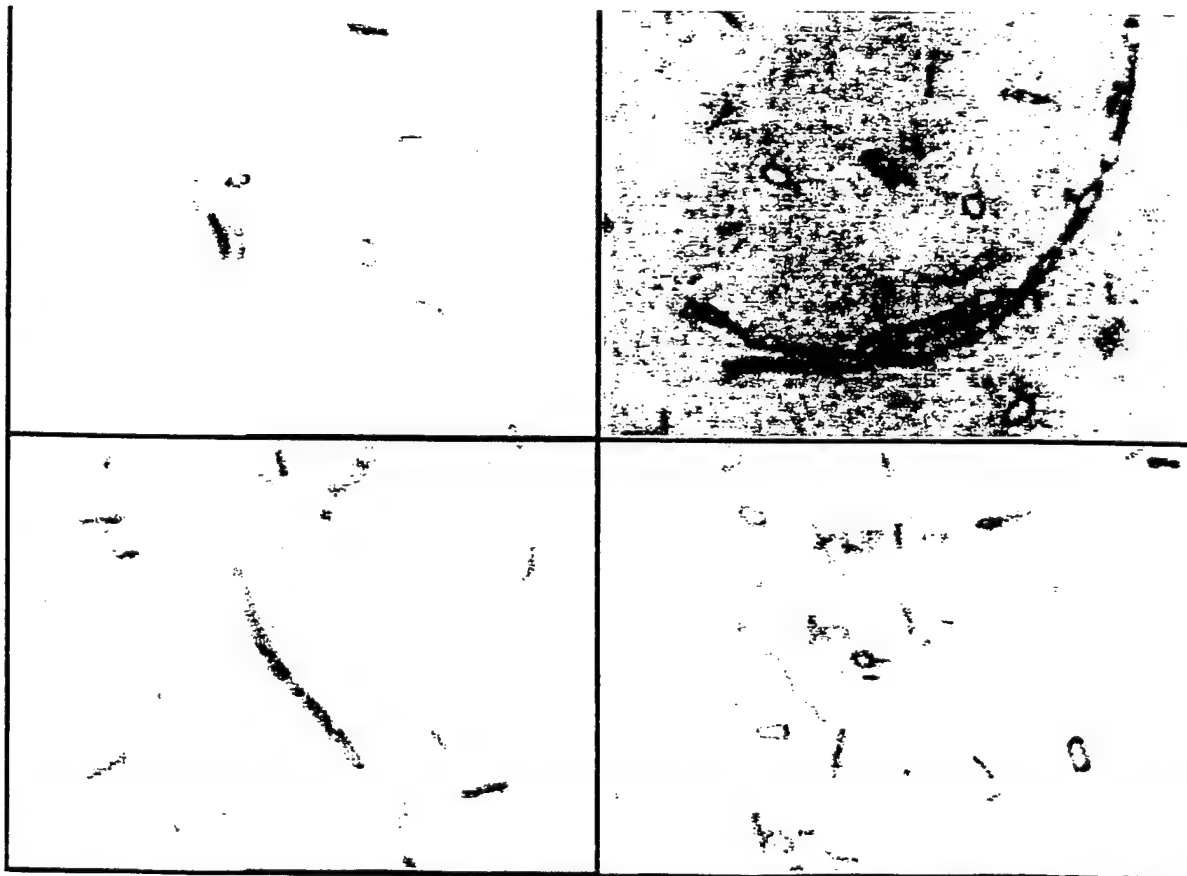
1c



7/20

1d

liquid  
from  
non-CB  
roll tubes  
from  
CB  
liquid  
cultures  
green  
stain

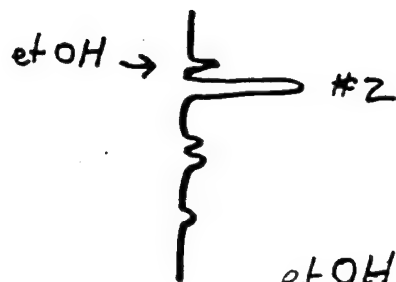


JS

11

# Figure 2

a)



etOH + Canada Balsam

± medium  
± cells

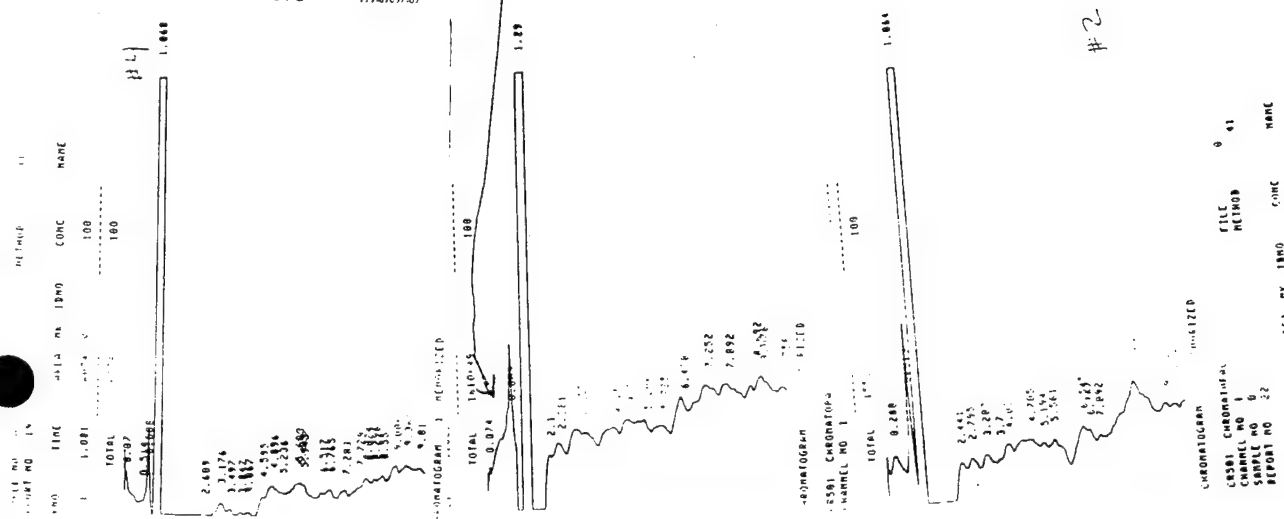
.003 % CB ~1:1:1:1  
.03 % CB ~3:4:2:2

b)

cells AC21

cells AC21  
+CB +etOH

cells AC21  
+ etOH



# Characterization of the Microbial Community Associated with the Shipworm, *Lyrodus pedicellatus*

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## Abstract

Terenid bivalves, or shipworms, utilize a microbial symbiotic community to digest the wood on which they feed. A cellulolytic nitrogen-fixing bacteria has been identified; but many other bacterial forms that dwell within the shipworm. This study was initiated to characterize other bacterial species within the shipworm, and to delineate regions in which they occur. A diagnostic stage was used to guide the enrichment and isolation stage. The diagnostic stage consisted of microscopic analysis, *in situ* hybridization with oligonucleotide probes, and microelectrode analysis for the determination of internal dissolved oxygen and pH. Microscopic analysis revealed a diversity of prokaryotes and eukaryotes, although their occurrence was restricted to the gill structure. Oligonucleotide probes revealed members of the flavobacteria group in the gill structure. Microelectrode analysis showed anaerobic conditions within all organs tested. Enrichments proceeded on a variety of media under both aerobic and anaerobic media. Many colonies formed on all media used, from which four strains were isolated. Tentative identification was possible for only one of the strains, which was enriched under sulfate reducing conditions. Characteristics of this strain are similar to those of *Desulfovibrio* spp. Additional characterization is necessary before identification of the other strains is possible.

## Introduction

The occurrence of symbiotic relationships between bacteria and a eukaryotic host is common. Among the most intensely-studied symbioses are those between cellulose-ingesting animals and consortia of cellulolytic bacteria. Well-characterized examples include ruminant mammals or termites and their respective cellulolytic microbial partners. Less comprehensively studied are marine wood-ingesting terenid bivalves, commonly known as shipworms. These molluscs exhibit radically modified morphological features that allow for the exploitation of wood as habitat; however, it was not accepted until recently that wood serves as the shipworm's chief, or perhaps only, source of nutrition.

The utilization of wood as a nutritional source is performed not without considerable digestive adaptation and specialization. Wood is composed largely of cellulose, a linear array of D-glucopyranose units linked by  $\beta$  (1-4) glycosidic bonds. Utilization of cellulose demands a suite of cellulolytic enzymes. Typically, eukaryotic organisms are bereft of these enzymes, and must rely on the cellulolytic activity of symbiotic bacterial consortia. The hypothesis that bacteria aid in the cellulolysis of wood ingested by shipworms was supported by Waterbury *et al.*'s (1983) isolation of a cellulolytic and nitrogen-fixing bacteria from *Lyrodus pedicellatus*. This bacterium occurs in dense concentrations within a modified gill structure called the Gland of DeShayes.

Many aspects of the bacterial community remain unclear despite the attention directed at the organism residing in the Gland of DeShayes. For example, bacteria have been observed in other organs, e.g., the appendix, and microscopic examination reveals a diversity of microorganisms in the Gland of DeShayes beyond the well-characterized

organism. Little is known about the phylogenetic identity or physiological activity of these microbes, i.e., whether or not a secondary group of bacteria is present that metabolize the cellulose breakdown products that are generated in the primary cellulolysis. Documentation of the presence of secondary metabolism, e.g., the presence of sulfate reducing or methanogenic bacteria, would support the hypothesis that, instead of a single cellulolytic bacterium, a true cellulolytic consortia is present in shipworms.

I propose to combine molecular techniques with appropriate enrichment methods to gain insight into the nature of the microbial community within the digestive system of *L. pedicellatus*. *In situ* hybridization will be employed on homogenates of the two organ systems known to harbor bacteria, namely the Gland of DeShayes and the caecum. Oligonucleotide probes will be selected to identify specific phylogenetic and physiological groups known to be participatory in cellulolysis. Enrichments for members of the *Bacteroides* group, sulfate reducers, members of the *Spirillum* group will be made on whole animal homogenates and specific media. Non-specific media will also be used to detect other symbionts. Both aerobic and anaerobic enrichments will be performed. Identification and characterization of isolated cultures will be evaluated by the outcome of a variety of diagnostic tests, e.g., determination of cellulolytic activity, presence of flagella using DIC microscopy. The outcome of molecular techniques and characterization of isolated bacteria will result in broader understanding of the microbial community within the shipworm.

## Materials and Methods

### I) Shipworms

Shipworms (*Lyrodus pedicellatus*) were obtained from a culture maintained in the laboratory of Dr. John Waterbury at Woods Hole Oceanographic Institute, Woods Hole, MA. Cultured animals were maintained in pieces of unidentified wood, into which the shipworms had bored and formed calcareous tubes. Wood pieces containing shipworms were immersed in a circulating seawater table ( $T \approx 20^\circ\text{C}$ ) at all times during the course of the study. As needed, shipworms were carefully extracted from wood in order to keep the tissues intact.

### II) Microscopy

Select organs from freshly sacrificed animals were excised and squashed on microscope slides. Sterile techniques were employed to prevent the introduction of exogenous microorganisms in this and in all other procedures. Phase-contrast microscopy was used to determine the presence and diversity of microorganisms within various organs. The presence of methanogens was tested visually by F420 autofluorescence.

### III) *in situ* Hybridization

Organs known or thought to harbor microorganisms (i.e., the Gland of Deshayes and the appendix, respectively) were independently excised from three shipworms and placed into individual sterile Eppendorf centrifuge tubes containing 1 ml 90:10 ethanol:formalin (v:v) fixative. Contents of each tube were separately transferred to sterile tissue grinder and homogenized completely. Tubes were spun for 5 min at 3 K, the supernatant discarded, and the pellet resuspended in 1.5 ml of a 50:50 (v:v) solution of storage buffer:95% ethanol, and maintained at  $-20^\circ\text{C}$  until the initiation of the hybridization procedure. The storage buffer consisted of 40 mM Tris at pH 7.5 and 0.2% Nonidet P40.

The hybridization procedure followed the methods of Nierzwicki-Bauer using a variety of diagnostic oligonucleotide probes (Table 1).

Table 1. Oligonucleotide probes used in *in situ* hybridization procedure to identify endosymbiotic bacteria within the shipworm *Lyrodus pedicellanus*.

Probe	Purpose/Target
Primer A	(+) Control, universal probe
Gamma subdiv.	id. of cellulolytic N <sub>2</sub> -fixing bacteria
Flavobacteria	id. of additional cellulolytic bacteria, e.g., <i>Bacteroides</i>
Archaea	id. of methanogenic bacteria
SRB	id. of sulfate reducers
Delta subdiv.	id. of sulfate reducers
High G + C	distinguish between low and high G + C
Low G + C	same as above
Bacteria	distinguish between archaea and bacteria

#### IV) Microelectrodes

Microelectrodes were used to measure *in vivo* dissolved oxygen concentration and pH according to Revsbech and Jørgensen (1986). A freshly extracted shipworm was placed on an agar plate below the microelectrode apparatus. Cross-sectional measurements were made on several organs by mechanically lowering the respective microelectrode through the tissue at 0.5 mm increments. I used microelectrodes modified according to Revsbech (1989). Dissolved oxygen and pH depth profiles were plotted for each organ examined.

#### V) Isolations/Enrichments

A number of media were used to isolate symbiotic bacteria from *L. pedicellanus* (Table 2).

Table 2. Media used to isolate endosymbiotic bacteria within the shipworm *Lyrodus pedicellatus*.

Media	Matrix (agar/liquid)	Target bacteria	Aerobic/anaerobic
Sea Water Complete	agar	aerobic heterotrophs/ bioluminescent bacteria	aerobic
MCTY	agar	spirochaetes/ <i>Spirillum</i>	aerobic/anaerobic
TYG	agar	<i>Bacteroides</i> group	anaerobic
NB	agar	spore formers	aerobic/anaerobic
SRB	liquid	sulfate reducers	anaerobic

Two to three intact shipworms were used for each media type. Animals were extracted from tubes and placed into an ethanol-flame-sterilized tissue grinder that contained 1 - 3 ml sterile seawater and thoroughly homogenized. For agar plates, sufficient volume (e.g., 25 -50 ul) homogenate was spread across the agar surface. Shipworm homogenate was heated at 75°C for 10 min prior to application to NB agar plates for isolation of endospore-forming bacteria. For inoculation of liquid SRB media, shipworms were homogenized in an anaerobic hood and drawn into an N<sub>2</sub>/CO<sub>2</sub> sparged 5 ml syringe. The syringe was sealed before removal from the hood. Three sealed SRB serum bottles received 1 ml of shipworm homogenate. One received 0.3 ml Na-lactate, and one received 0.3 ml Na-acetate, for a final concentration of 10mM for both carbon sources. The final serum bottle received no additional carbon source and served as a control.

Inoculates were checked for growth daily. As colonies appeared on plates, they were examined microscopically and re-streaked in order to obtain pure cultures. Diagnostic tests were performed on pure cultures to determine the phylogenetic identity and physiological activity of the bacteria.

## Results

### Microscopy

Initial microscopic examination of squashed organs revealed differences in the relative abundance of microorganisms among various organs of *L. pedicellatus*. No organisms were observed in squashes prepared from the appendix or the mid-gut region. The gill structure harbored an abundance of microorganisms, including ciliated eukaryotes (Figure 1) and a variety of bacteria (Figure 2). F420 fluorescence was exhibited by several individual eukaryotic ciliates but not by bacterial cells.

### *in situ* Hybridization

The slide incubated at 37°C was accidentally exposed to light at room temperature for approximately five hours after the initial probe application. This slide was washed, the probes re-applied, and the incubation re-initiated at 37°C in the dark.

Positive controls were conducted on cells from several putatively identified pure cultures, with inconsistent results. An abundance of wood particulate material was present in the appendix slide preparation, and eukaryotic cellular material occurred in the gill slide preparation. Resolution of fluorescence varied among the probes applied to shipworm



samples (Table 3). For example, universal Primer A (37°C) did not fluoresce at all, whereas universal Primer C (45°C) fluoresced when applied to the gland sample but not the appendix sample. Clearly visible fluorescent cells were visible in gland samples that were treated with Archae 291 (Figure 3, middle row, left panel),  $\beta$  (Figure 3, middle row, middle panel) and flavobacteria (Figure 3, bottom row, right panel) probes. The flavobacteria probe showed the most intense and widespread fluorescence. Many fluorescent cells were embedded within eukaryotic structures, although this was not effectively captured on film. No fluorescence was observed in the appendix samples.

Table 3. Results of *in situ* hybridization with oligonucleotide probes applied to samples prepared from the gill structure and appendix from the shipworm *Lyrodus pedicellatus*.

Sample	Hi G+C	Lo G+C	Arch 291	$\alpha$	$\beta$	$\gamma$	$\Delta$	Flavo
Appendix	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Gland	(-)	(-)	+	(-)	+	(-)	(-)	++

Designation: (-) = no fluorescence. + = detectable fluorescence at 0.5 second integration ++ = detectable fluorescence at 0.25 second integration

### Micro-Electrode Analysis

A heartbeat was observed in the organism used for micro-electrode analysis. Respiration continued throughout the analysis, which proceeded for approximately one hour. Every organ that was probed, i.e., gill structure, appendix, brood pouch, exhibited anaerobic conditions immediately below the tissue surface. Oxygen was detected in the agar below organism once the probe had passed completely through the tissue. pH ranged from 7.6 to 8.0, and did not differ significantly from the exterior environment.

### Enrichments

Aerobic enrichments on SWC plates yielded a variety of colony forms. For the most part, growth occurred within two days. However, some colony forms did not become apparent until two weeks after the initial inoculation. Colony morphotypes ranged from round, raised, and glossy colonies to flattened, amorphous, spreading colonies. Several highly pigmented colonies were present, with colors ranging from yellow to red. Component cells exhibited a range of shapes, including short motile rods, long non-motile rods, and non-motile curved rods. Aerobic enrichments on MCTY agar plates yielded amorphous, loose colonies that were initially difficult to separate. Preparation of plates with higher agar content yielded better separation of colonies.

A strong, putrescence-like odor emanated from the anaerobic Gas Pak upon opening. Anaerobic enrichments on TYG plates exhibited rich, amorphous growth. Re-streaking was attempted, but the catalyst was not replaced upon resealing the Gas Pak container, and anaerobic conditions were not achieved. Many of the re-streaked plates showed no growth. Microscopic examination of constituents from the initial enrichment plate exhibited a broad array of microbial forms, including cocci, non-motile rods with dipolar, bulbous terminations, vibrios, and long narrow rods.

Turbidity increased in the SRB Na-lactate and Na-acetate serum bottles relative to the control within four and seven days, respectively. A variety of microbes were present in both bottles. The lactate bottle exhibited more abundance and diversity; cell types included vibrios, small cocci, and irregularly shaped rods. Several forms of bacteria were present in



the acetate enrichment, as was a eukaryotic flagellate (Fig. 4). Interestingly, the flagellate remained active under anaerobic conditions for at least thirteen days. The lactate and acetate bottles produced 3.8 and 1.4 mM sulfide, respectively, indicating that sulfate was reduced with both carbon sources. Agar deeps were prepared from the lactate enrichment, but not from the acetate enrichment because of its slow development.

Table 4. Characteristics of colonies isolated from the shipworm, *Lyrodus pedicellatus*.

Designation	Media	Aerobic/Anaerobic	Colony Description
Yellow Cluster	SWC agar plate	Aerobic	Small, glossy, raised, bright yellow pigmentation
Pale White	SWC agar plate	Aerobic	Flat, diffuse, spreading, pale white
Long-Curved	MCTY agar plate	Aerobic	Flat, amorphous, spreading, white
SRB Vibrio	SRB shake tube	Anaerobic	Lens-shaped, light brown

### Isolations

Isolated colonies from the first re-streak of initial enrichments on agar plates yielded three pure cultures, two on SWC agar plates and one on MCTY agar plates. Three morphologically distinct colonies on SWC were repeatedly re-streaked, but pure cultures were not achieved despite the consistent occurrence of isolated colonies on re-streaked plates, i.e., the isolated colonies always consisted of a mixed culture.

Pure cultures grown on SWC agar plates exhibited two different colony morphotypes, one yellow, glossy, and circular and the other pale white, flattened, and amorphous (Table 4). Microscopic examination revealed curved rods, sometimes with bulbous ends, in the yellow-pigmented colonies (Table 5, Figure 5 upper left; Figure 6). These cells were immotile, gram negative, oxidase negative, and catalase positive (Table 5). Growth, albeit minor, occurred on cellobiose, carboxy-methyl cellulose, and xylase, but not on cellulose. They could not grow anaerobically, nor did they form spores upon pasteurization or exhibit fluorescence.

The pale white colonies were composed of long dark cells that exhibited non-refractile areas at various points along the length (Fig. 7). DIC microscopy did not reveal the nature of the refractile areas. These motile rods were gram negative, oxidase and catalase positive (Table 5), and did not grow on cellulose or cellulose breakdown-products (Table 6).

One pure culture that formed amorphous, flattened white colonies was obtained from MCTY agar plate inoculations (Table 4). These gram negative cells were irregularly rod-shaped, motile, and scored positive for the oxidase and catalase tests (Table 5). Logistical restraints prevented any further characterization.

Isolated colonies were present within four days in all SRB Na-lactate agar deep dilutions (i.e.,  $10^3$  to  $10^9$  original concentration). The colonies were brown and lens-shaped. Microscopic examination showed the cells to be rapidly-motile vibrios. A single polar flagellum was observed under DIC microscopy.

Table 5. Cell characteristics from pure colonies isolated from the shipworm *Lyrodus pedicellatus*.

Designation	Cell Description	Motility	Gram Stain	Oxidase Test	Catalase Test
Yellow Cluster	Curved rods, sometimes with bulbous end	(-)	(-)	(-)	(+)
Pale white	Long rods, always featuring non-refractile transparent elements	(-)	(-)	(+)	(+)
Long-Curved	Long irregularly-shaped rods	(+)	(-)	(-)	(+)
SRB Vibrio	Vibrio	(+): SP <sup>1</sup>	ND <sup>2</sup>	ND	ND

SP<sup>1</sup>: single polar flagellum as determined by DIC microscopic analysis  
ND<sup>2</sup>

## Discussion

Enrichment techniques and *in situ* hybridization demonstrated a broad diversity of microorganisms within the shipworm *Lyrodus pedicellatus*. Although only four bacteria were isolated in pure culture, enrichments yielded a variety of organisms under aerobic and anaerobic conditions using several types of enrichment media. At least one pure culture was obtained from an anaerobic sulfate reducing culture. Three other pure cultures were isolated from aerobic agar plates. *In situ* hybridization tentatively indicated the presence of archaeobacteria, members of the  $\beta$  subclass, and flavobacteria.

Waterbury *et al.* (1983) isolated and characterized a cellulolytic, nitrogen fixing bacteria from the Gland of DeShayes of *L. pedicellatus*. This bacterium was gram (-), moved via a single polar flagellum, and exhibited morphological pleomorphy, appearing either as a spiraled or long straight rod (Waterbury *et al.* 1983). The only bacterium isolated in the present study that matches these characteristics is the irregularly-shaped rod grown on MCTY agar plates. Further characterization (i.e., demonstration of cellulolytic activity, 16 s rRNA *in situ* hybridization) of this isolate would be necessary before making any further conclusions about its identity. None of the other bacteria isolated in this study share the characteristics of the cellulolytic rod identified by Waterbury *et al.* (1983). Of these, putative identification is possible only for the vibrio isolated from the SRB enrichment. The identity of the curved rod that formed yellow colonies and the long rod that formed flattened, pale-white colonies remains unclear.

The sulfate-reducing bacteria appears to be *Desulfovibrio* spp. This bacteria was putatively identified based on its vibrio shape, single, polar flagellum, and its presence in lactate and absence in acetate (Waddle and Bak 1992). To my knowledge, this is the first report of an anaerobic, sulfate-reducing bacterium isolated from any terenid bivalve. Caution must be exercised when considering the role of SRBs within the shipworm. For example, it is difficult to distinguish between resident and transient microorganisms within marine organisms (Harris 1993). Given that the woodworm creates an aerobic environment within its burrow, shows anaerobic conditions within all organ systems, and because the organisms were thoroughly rinsed with sterile seawater prior to homogenization, it is reasonable to accept that *Desulfovibrio* spp. did originate within the shipworm. This still does not clarify its status, i.e., transient vs. resident. Identification of other participants of cellulolytic symbiotic systems may support the role that *Desulfovibrio* spp. is surviving at the expense of by-products of cellulolysis.

In addition to the variety of bacteria described, several eukaryotic organisms were observed within the shipworm. Although characterization of eukaryotic organisms is outside the scope of this study, their presence is nonetheless notable because of the absence of documentation of such symbiotic organisms. One ciliate that survived under anaerobic conditions exhibits external morphological resemblance to a protist occurring in the termite hindgut (Joy Sabl, personal communication).

To summarize, a number of microorganisms, both prokaryotic and eukaryotic, were observed within the shipworm, *L. pedicellatus*. Four bacteria were brought into pure culture, including what appears to be *Desulfovibrio* spp. The identity of the remaining isolates, and indeed much of the microbiota of *L. pedicellatus*, remain unclear.

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## Figure Legends

Figure 1. Phase contrast micrographs of eukaryotic organisms observed within the gill structure of the shipworm, *Lyrodus pedicellatus*. Sample was obtained from a freshly sacrificed specimen, and squashed on a microscope slide.

Figure 2. Phase contrast micrographs of bacteria and eukaryotic organisms observed within the gill structure of the shipworm, *Lyrodus pedicellatus*. Sample was obtained from a freshly sacrificed specimen, and squashed on a microscope slide.

Figure 3. Phase contrast micrographs of oligonucleotide probe fluorescence applied to homogenated samples of shipworm (*Lyrodus pedicellatus*) gill structure. Three panels show fluorescent cells: Archae 291 probe (middle row, left panel),  $\beta$  probe (middle row, middle panel), and the flavobacterium probe (bottom row, right panel). The other panels show extraneous material that non-specifically bound the probe.

Figure 4. Phase contrast micrograph of microbiota present in acetate SRB enrichment bottle after thirteen days. SRB serum bottle was inoculated with anaerobically-prepared homogenate of the shipworm *Lyrodus pedicellatus*.

NOTE: FIGURE 8 IS OUT OF ORDER

Figure 5. Phase contrast micrograph of bacteria from isolated colonies on SWC agar plates. Original inoculate was homogenized shipworm (*Lyrodus pedicellatus*). Upper left panel shows curved rods designated as "Yellow Cluster", and lower right panel shows long rods designated "Pale White". Note the presence of non-refractile features of Pale White cells.

Figure 6. DIC micrograph of a pure culture isolated from the shipworm *Lyrodus pedicellatus*. Isolate is designated "Yellow Cluster". Note the presence of cells exhibiting bulbous ends, i.e., middle of upper right panel.

Figure 7a. Figure 6. Phase contrast micrograph of a pure culture isolated from the shipworm *Lyrodus pedicellatus*. Isolate is designated "Pale White". Note the presence of non-refractile features common to all cells.

Figure 7b. DIC micrograph of a pure culture isolated from the shipworm *Lyrodus pedicellatus*. Isolate is designated "Pale White". Note the presence of non-refractile features common to all cells.

Figure 8. TOP: Phase contrast micrograph of a pure culture isolated from the shipworm *Lyrodus pedicellatus*. Culture was isolated under sulfate reducing conditions using lactate as a carbon source. BOTTOM: DIC micrograph of cells in SRB-lactate isolation. Note the single polar flagellum extending from each cell

FIGURE 1.

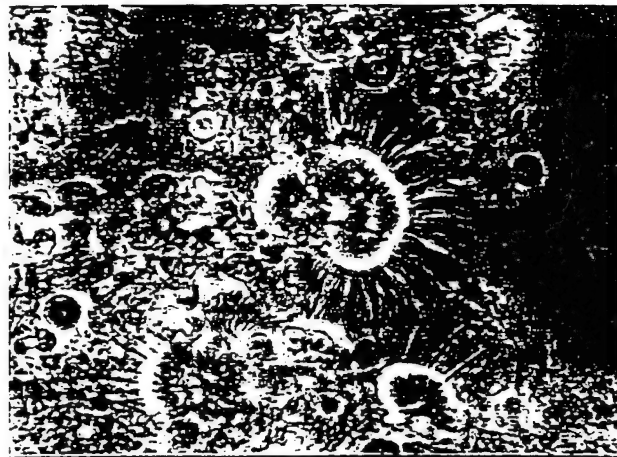
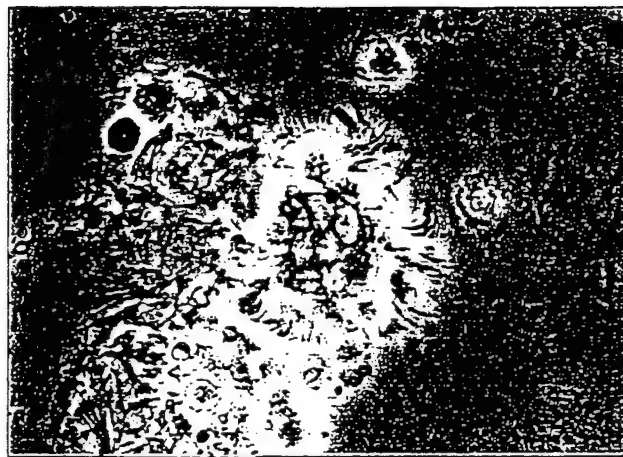
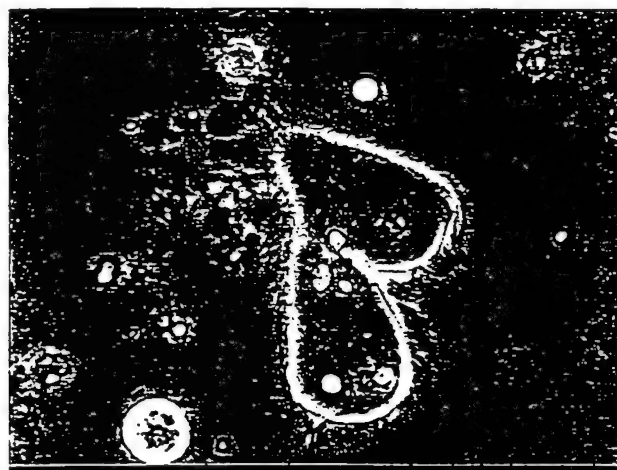
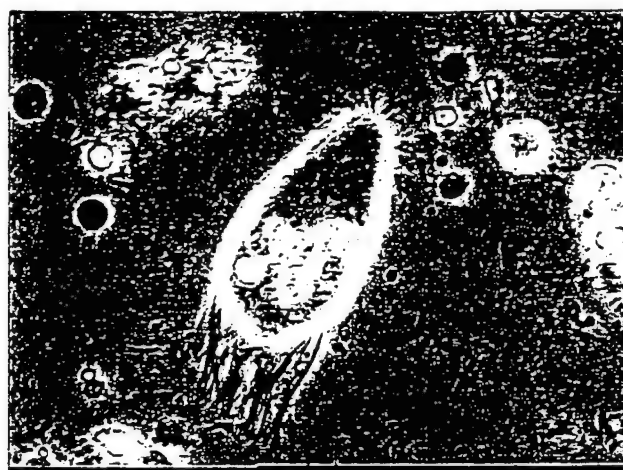
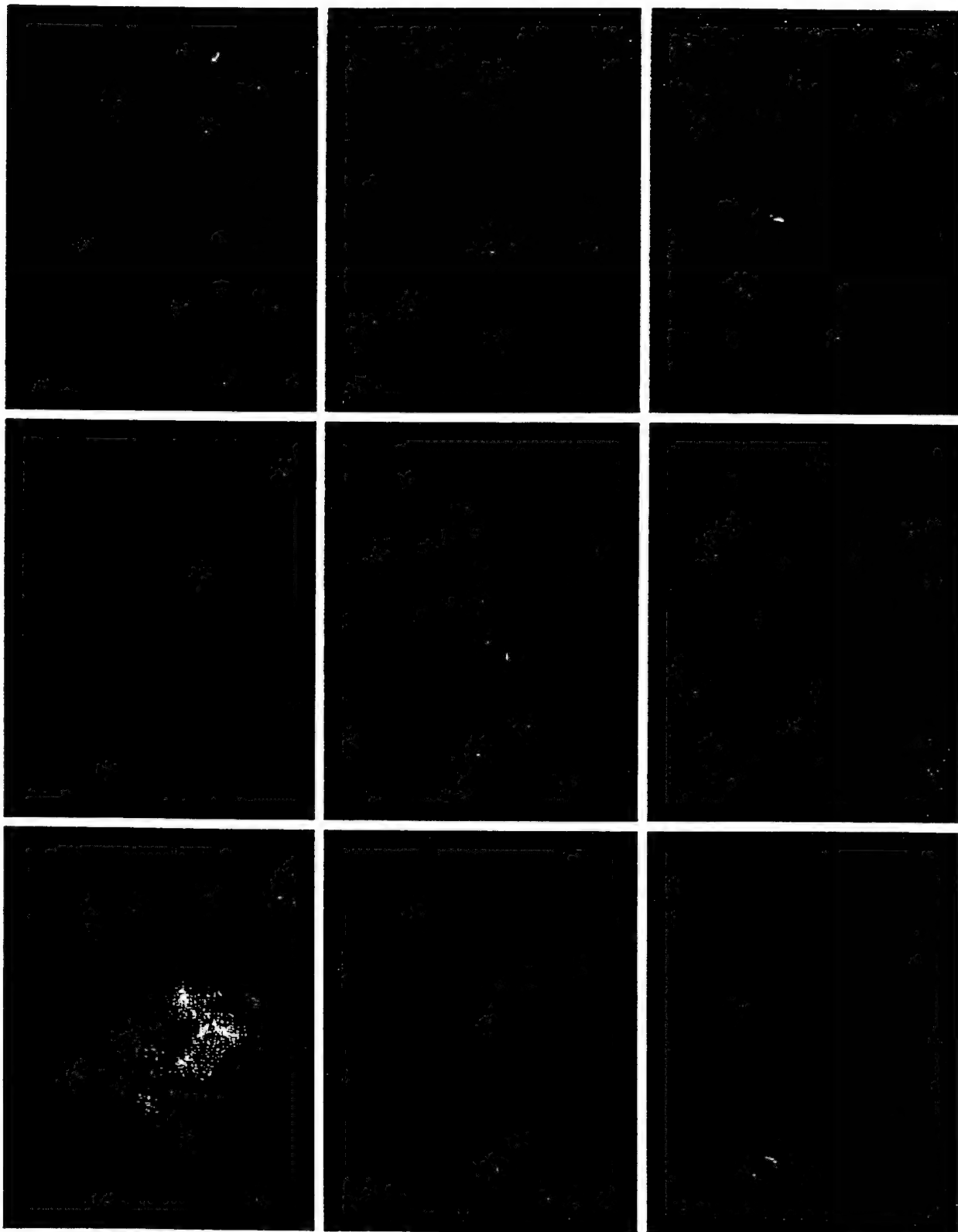


FIGURE 2.



Figure 3.



Top

MIDDLE

Bottom



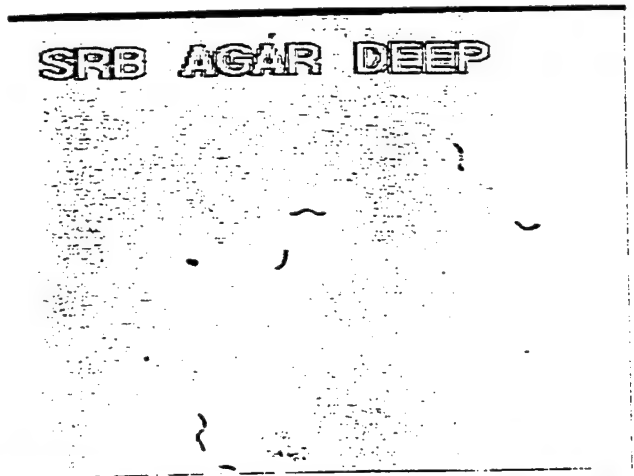
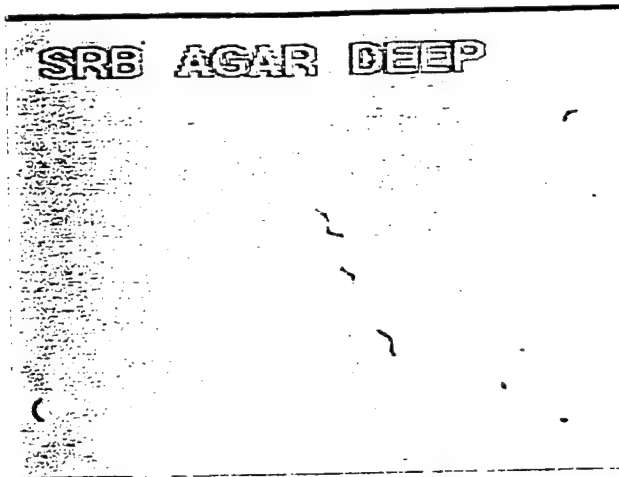


Figure 8.

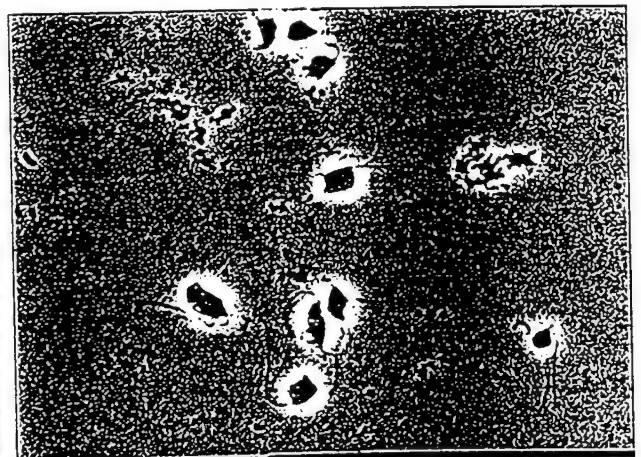
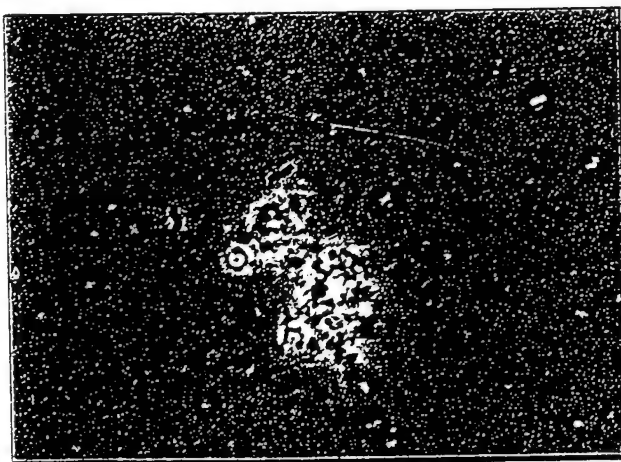
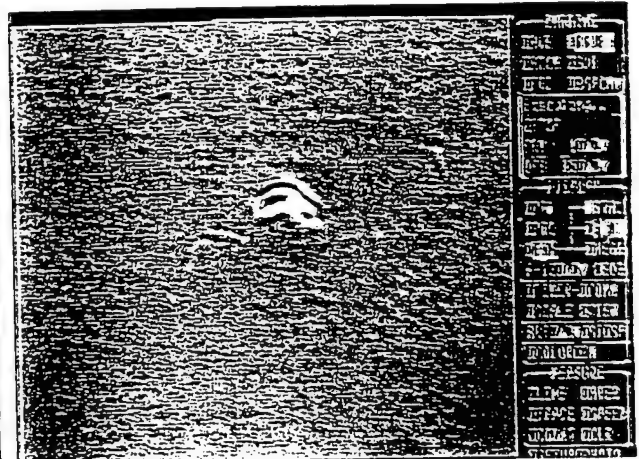
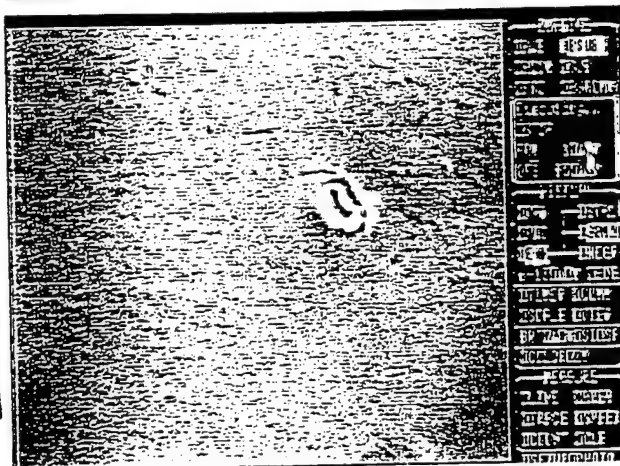
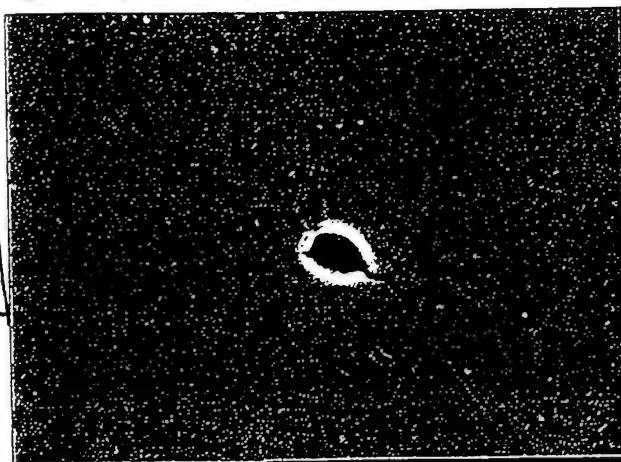


FIGURE 4.



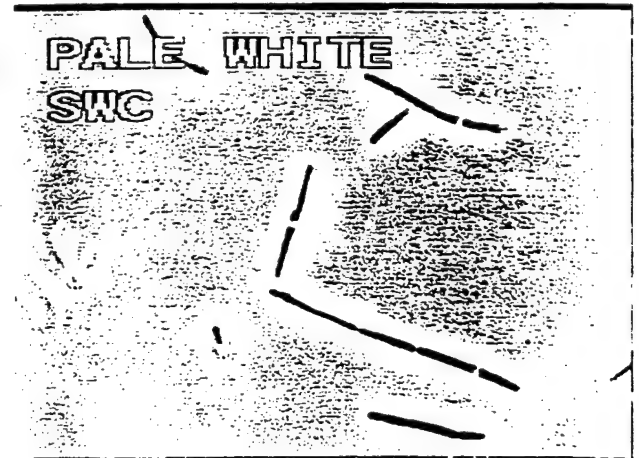
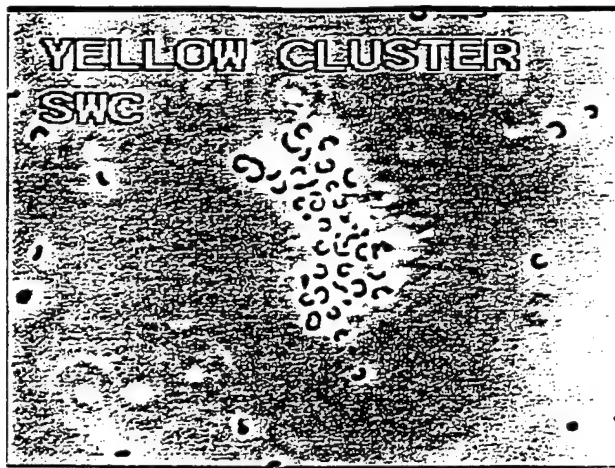


Figure 5.

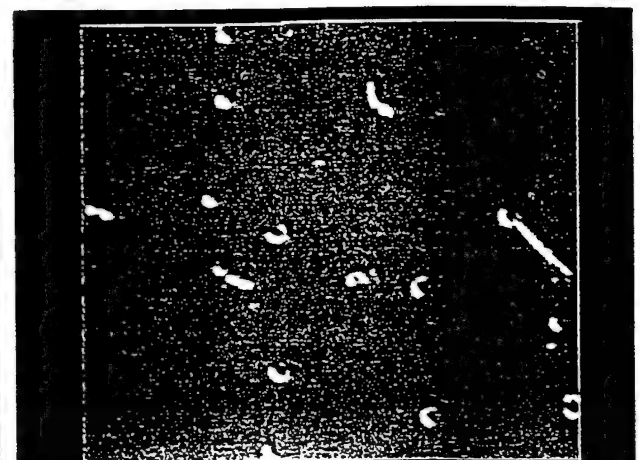
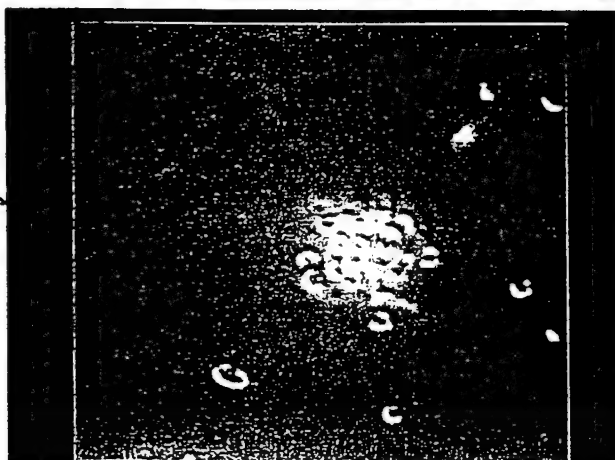
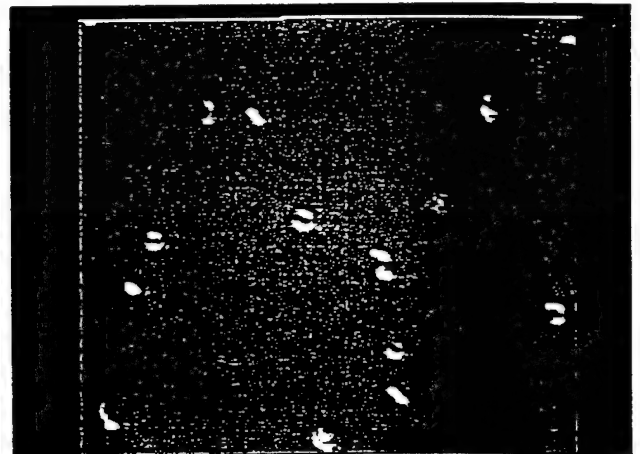
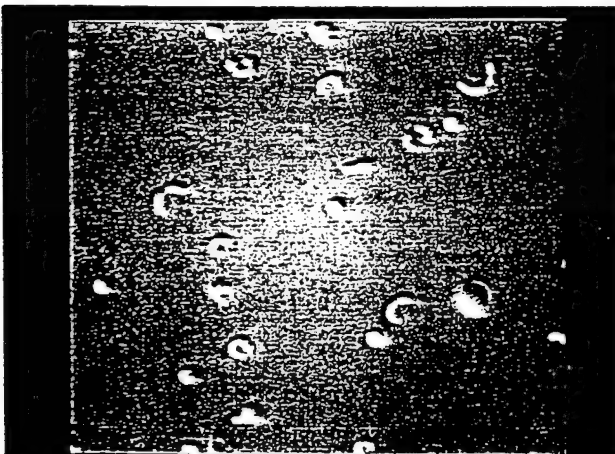


Figure 6.



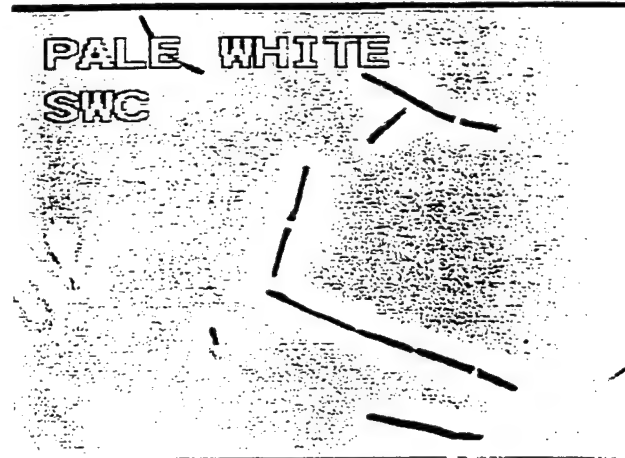
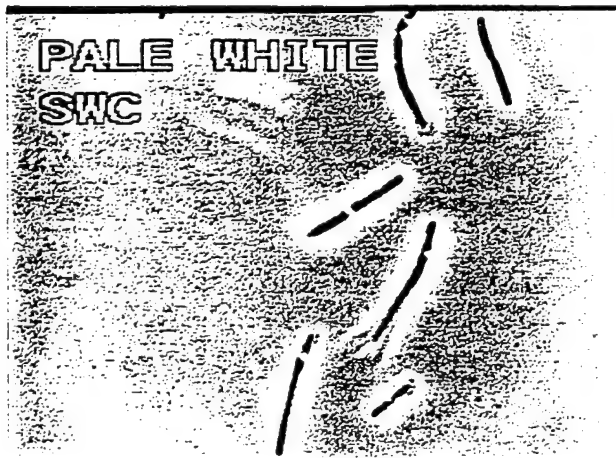
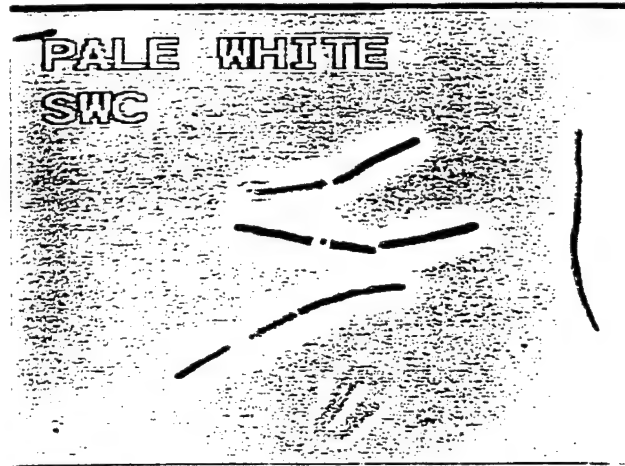
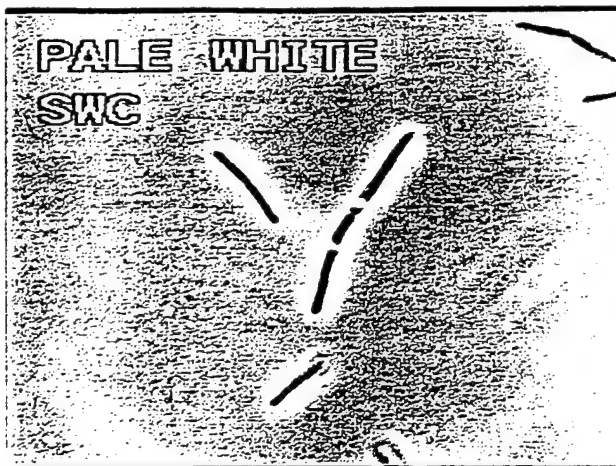


Figure 7a.

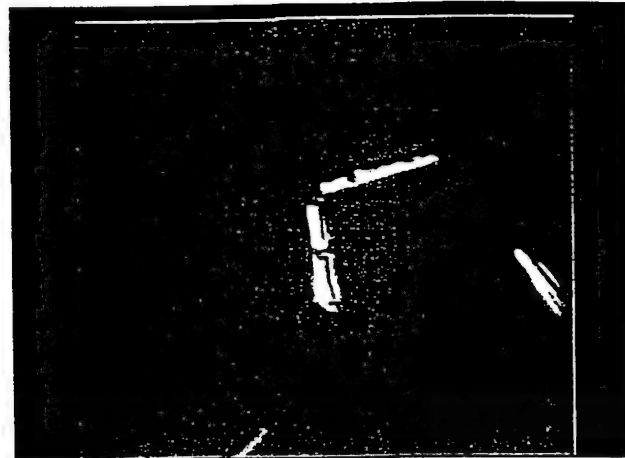
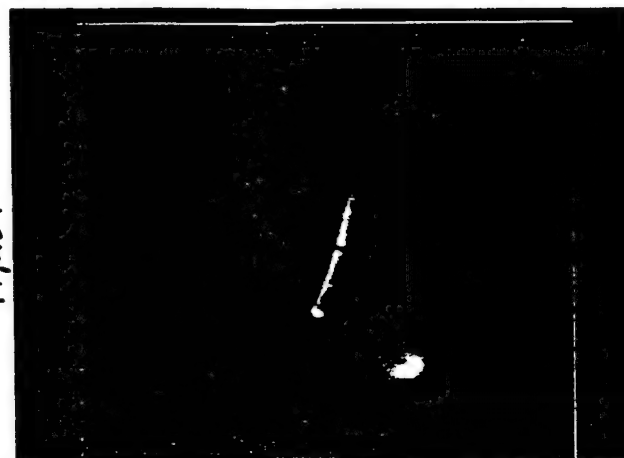
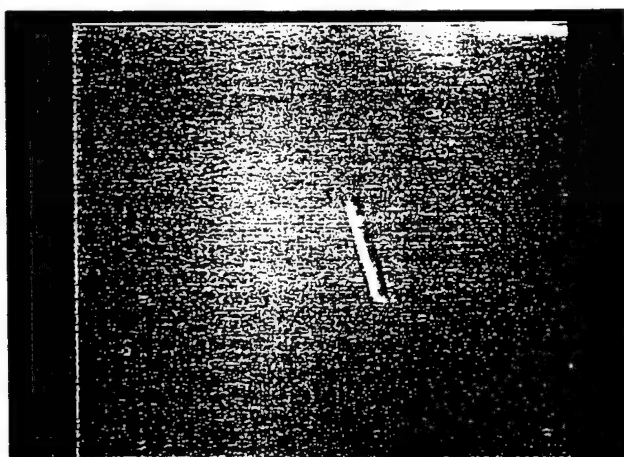


Figure 7b.

# THE PHYSIOLOGY OF N<sub>2</sub> FIXATION BY NON-HETEROCYSTOUS CYANOBACTERIA

INDIVIDUAL PROJECT WORK - MICROBIAL DIVERSITY COURSE 1995

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## INTRODUCTION

Restricted to procaryotes in general, the ability to fix dinitrogen (diazotrophy) is universal among those cyanobacteria that produce heterocysts. It is much less widespread among non-heterocystous cyanobacteria. Most species of non-heterocystous cyanobacteria examined so far fix N<sub>2</sub> only during the dark phase of a dark-light cycle and some additionally require microaerobic conditions. Among unicellular cyanobacteria *Gloeotheca* spp., *Synechococcus* SF-1, *Cyanothece*, *Aphanothece* and *Synechocystis* were reported to fix N<sub>2</sub>. In a case of filamentous non-heterocystous cyanobacteria, aerobic N<sub>2</sub> fixation has been reported in several strains of *Oscillatoria* including *Trichodesmium* spp. and *Microcoleus chthonoplastes*. However, *Trichodesmium* species differ substantially from all other non-heterocystous N<sub>2</sub> fixers. They exhibit light-dependent N<sub>2</sub> fixation in highly oxygenated environments.

Most studies on N<sub>2</sub> fixation by non-heterocystous cyanobacteria have centered on those organisms that can fix N<sub>2</sub> aerobically. One reason for this particular interest in aerobes is that a current goal of agricultural research is to transfer functional N<sub>2</sub> fixation (*nif*) genes to the chloroplasts of higher plants, where ATP and reductant are readily available. It is widely considered that cyanobacteria resemble the evolutionary ancestors of contemporary chloroplasts, so study of aerobic N<sub>2</sub> fixation in non-heterocystous, especially unicellular cyanobacteria seems to be the most relevant.

Nitrogenase (N<sub>2</sub>ase) is a complex enzyme with remarkably similar properties in about 25 organisms in which it has been investigated. It consists of 2 iron-sulphur proteins, that individually, have no detectable enzymatic activity. Together they catalyse the reduction of variety of substrates, i.e. N<sub>2</sub>, N<sub>3</sub><sup>-</sup>, N<sub>2</sub>O, HCN, C<sub>2</sub>H<sub>2</sub>, CH<sub>3</sub>NC or H<sub>3</sub>O<sup>+</sup>. The lack of the strict specificity towards N<sub>2</sub> allowed development of a relatively straightforward assay of N<sub>2</sub>ase activity: the acetylene reduction assay combined with the use

of gas chromatography. Additional advantage of the method is the fact of using intact (i.e. fully viable) cells in the assay.

The primary goal of the project is to test some features of a cyanobacterial strain classified as *Microcoleus* spp.. Preliminary tests of N<sub>2</sub> fixation (done at the lab from which I got the strain, Amsterdam, The Netherlands) suggest that the strain fixes N<sub>2</sub> in a similar way as *Trichodesmium*. In order to do the N<sub>2</sub> fixation assay on *Microcoleus* spp. and compare it with a few chosen cyanobacterial strains, some additional experiments need to be done:

1 - *Microcoleus* spp. is not axenic.

Learning of purification procedures of cyanobacterial strains from a natural environment (the Sippewissett Salt Marsh, Woods Hole, Mass.) at the course is applied for the purification of *Microcoleus* spp..

2 - *Microcoleus* spp. grows in the form of big, compact aggregates, which are not disrupted by vortexing. From the literature data it is known, for example, that in order to get fine suspension cultures of higher plants, a commercially available composition of cellulolytic enzymes is added to the medium. Since it is not known which enzymes can weaken *Microcoleus* spp. sheaths and cell walls, an attempt to find a bacterial strain capable of digesting cell walls of *Microcoleus* spp. will be made. However, a mixture of a few cellulolytic enzymes will also be made and tested.

3 - Assay of N<sub>2</sub> fixation by acetylene reduction.

The following strains will be tested first: *Synechococcus* WH 8902, *Synechococcus* WH 8904 and *Trichodesmium* NC. The *Microcoleus* spp. strain will be tested after purification. Then the N<sub>2</sub> fixation pattern of *Microcoleus* spp. will be compared with the ones of the above strains.

4 - The strain was named *Microcoleus* based on its morphology. Sequence comparison of some regions of 16S rRNA of such cyanobacteria as *Microcoleus chthonoplastes*, *Lyngbya*, *Oscillatoria* and *Synechococcus* WH 8904 with the respective sequence of *Microcoleus* spp. should reveal the identity of *Microcoleus* spp. The sequencing data can be used for other studies.

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## MATERIALS AND METHODS

### 1. PURIFICATION OF CYANOBACTERIAL STRAINS.

Samples of cyanobacterial mat were collected at the Sippewissett Salt Marsh, Woods Hole, Mass.. Pieces of the mat containing cyanobacteria were placed on the 2 x SNAX solid medium. Plates were checked every week and some filaments were transferred in a sterile manner onto the fresh solid 2 x SNAX medium.

*Microcoleus* spp. aggregates of 0.5 to 1.0 cm in size were placed on the 2 x SNAX solid medium. Every week filaments that glided away from the aggregate were picked up with the sterile flat niddle under the binocular microscope and transferred onto the fresh solid 2 x SNAX medium.

The composition of 2 x SNAX: 20 uM Na<sub>2</sub>CO<sub>3</sub>, 2.0 mM NaNO<sub>3</sub>, 200 uM NH<sub>4</sub>Cl, 20 uM K<sub>2</sub>HPO<sub>4</sub>, 0.2 ml Cyano Trace Metals, 3.0 uM EDTA, 750 ml filtered Sea Water, 250 ml double-distilled water.

### 2. MAKING A FINE SUSPENSION OF *MICROCOLEUS* SPP.

#### 2.1. BACTERIAL STRAIN ISOLATION

Isolation of *Myxobacteria* was done during the course classes (Peterson, 1969).

#### 2.2. TEST OF A CELLULOLYTIC ENZYMES MIXTURE

Three enzymes were used: Beta-glucuronidase from *Helix pomatia* type H1 (Sigma), Cellulase E.C. 3.2.1.4 from *Aspergillus niger* (Sigma) and Chitinase E.C. 3.2.1.14 from *Serratia marcescens*. The concentration of each enzyme in the stock solution was 0.05 g/ml. 100 ul and 250 ul of the stock was added per 50 ml of the *Microcoleus* spp. culture, respectively. Cultures were incubated in a growth chamber with 14-hour light and 10-hour dark cycle at 25 C.

### 3. ASSAY OF N<sub>2</sub> FIXATION BY ACETYLENE REDUCTION

20 ml of cyanobacterial culture was used as an inoculum for 100 ml of the liquid 2 X SO medium (2 x SNAX medium without NaNO<sub>3</sub> and NH<sub>4</sub>Cl). After 7 days 50 ml of the above culture was inoculated to 0.5 l of 2 X SO. The flasks were incubated at 25 C in a

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growth chamber with 14-hour light and 10-hour dark cycle for 3 days. Then the measurements were done as follows: first 24 hours 20 ml samples were taken every 6 hours (i.e. 12:00, 18:00, 24:00, 6:00, etc.). When the period of N<sub>2</sub> fixation was found, assays were done every hour starting at 3:00 am till 9:00 am.

Each 20 ml sample was placed into 60 ml bottle and closed with a rubber plug. 100% acetylene was injected through the plug to the final concentration 10% , so the gaseous phase above the sample was the mixture of acetylene and the air. 300 ul of the gaseous phase was taken at time 0 min. (control), 20 min., and 40 min.. Samples of 200 ul were applied to the POROPAK Q column of the SHIMADZU GC-14A gas chromatograph.

#### 4. DETERMINATION OF Chlorophyl a

After the acetylene reduction assay had been completed, each 20 ml sample was filtrated through the GF-4 glass fiber filter on the Millipore filtration system and frozen at -20C. Samples will be used for the chlorophyl a determination (next week) according the following procedure: each pellet will be grinded and extracted twice with 90% (V/V) methanol for 1 hr at 4C in dim light followed by centrifugation at 10.000 g for 10 min. at 4C. The chlorophyl a content is calculated from the absorbance of the methanolic extract at 665 nm using equation:

$$c \text{ [ug/ml]} = OD_{665 \text{ nm}} \times 13.9$$

(Tandeau de Marsac and Houmard, 1988)

#### 5. DNA EXTRACTION FROM CYANOBACTERIA (acc. A. Sghir, J. Dore, unpublished)

Cyanobacterial pellet from the 50 ml culture was digested in a resuspension buffer with Lysosyme, Pronase, Mutanolysin and RNase at 37 C for 1 hr. Then the extraction buffer was added and the incubation was continued for 1 hr at 37 C and for 30 min. at 55 C. DNA was extracted with phenol, chloroform and isoamyl alkohol (totally 6 consecutive extractions). Final aqueous phase was used for DNA precipitation. Precipitated DNA was stored at 4 C untill the use.

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## 6. MICRO PCR FOR rDNA-PCR OPTIMIZATION

The universal 16S rRNA primers were used. PCR was done in the standard PCR buffer. The volume of the reaction mix was 12.5 ul. PCR was initiated by so called "hot-start" procedure.

## 7. GEL ELECTROPHORESIS

PCR products were analysed by standard gel electrophoresis in agarose.

## RESULTS AND DISCUSSION

### 1. PURIFICATION OF *MICROCOLEUS CHTHONOPLASTES* FROM THE SIPPEWISSETT SALT MARSH AND *MICROCOLEUS* SPP.

Both strains were obtained as the pure cultures (Figure 1 and 2).

The *Microcoleus* spp. was free of bacterial contamination, however some additional tests should be done in order to be sure that the strain is axenic. The amount of the *Microcoleus* spp. is too little in order to start the planned studies. However, the strain is growing very well on the solid medium and soon can be used for propagation in the liquid medium.

The *Microcoleus chthonoplastes* cultures still have bacterial contaminants. The attempt to make them axenic will be done next week.

### 2. SUSPENSION CULTURES OF *MICROCOLEUS* SPP.

#### 2.1. BACTERIAL STRAINS ISOLATION

Some *Myxobacteria* were isolated. Since the time of the course has been finished, I have no time to test their ability to digest *Microcoleus* spp. cell walls.

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## 2.2. CELLULOLYTIC ENZYMES

Addition of a mixture of 3 cellulolytic enzymes to the *Microcoleus* spp. medium did not loosed the cell wall structure. The strain was continuing good growth in a form of big aggregates.

## 3. ASSAY OF N<sub>2</sub> FIXATION

The assay was completed for 2 unicellular marine cyanobacterial strains: *Synechococcus* WH 8902 and WH 8904. Both strains fix N<sub>2</sub> at THE END of the dark phase of 10-hour dark and 14-hour light cycle. In the case of *Synechococcus* WH 8902, N<sub>2</sub> fixation still rised during the first hour of of light period and then slowly decreased (Table 1). In the case of *Synechococcus* WH 8904, the decrease of N<sub>2</sub> fixation was already observed during the first hour of light (Table 2).

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Table 1. The pattern of the acetylene reduction of *Synechococcus* WH 8902  
(the Light/Dark cycle was 12 hr L (from 6:00 am) / 10 hr D (from 8:00 pm))

Day	Time	Hours passed from the first measurement	Area of the C <sub>2</sub> H <sub>4</sub> peak after	
			20 min.	40 min.
07 14	12:00 noon	0	4	23
	6:00 pm	6	4	5
	12:00 midnight	12	8	3
07 15	6:00 am	18	158	574
	12:00 noon	24	0	0
	8:00 pm	32	0	0
07 16	5:30 am	41.5	498	1746
	7:00 am	43	402	2475
	8:00 am	44	222	1508
07 17	9:00 pm	69	0	0
	12:00 midnight	72	3	0
07 18	3:00 am	75	33	157
	4:00 am	76	185	702

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Table 2. The pattern of the acetylene reduction of *Synechococcus* WH 8904  
(the Light/Dark cycle was 12 hr L (from 6:00 am) / 10 hr D (from 8:00 pm))

Day	Time	Hours passed from the first measurement	Area of the C <sub>2</sub> H <sub>4</sub> peak after	
			20 min.	40 min.
07 14	12:00 noon	0	0	0
	6:00 pm	6	0	0
	12:00 midnight	12	0	0
07 15	6:00 am	18	836	3538
	12:00 noon	24	36	53
	8:00 pm	32	21	0
07 16	5:30 am	41.5	606	1886
	7:00 am	43	601	1860
	8:00 am	44	173	431
07 17	9:00 pm	69	29	20
	12:00 midnight	72	0	1
07 18	3:00 am	75	195	796
	4:00 am	76	238	997

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#### 4. AMPLIFICATION OF 16S rRNA

1.5 kb fragment was amplified by PCR (Figure 3).

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- Peterson J., 1969. Isolation, cultivation and maintenance of the myxobacteria. In: Norris J. and Ribbons D. (eds.). Methods in Microbiology, vol. 3 B , pp. 185-210. Academic Press.
- Tandau de Marsac N. and J. Houmard. Complementary chromatic adaptation: Physiological conditions and action spectra. In: Packer L. and Glazer A. N. (eds.). Methods in Enzymology, vol. 167, p. 322. Academic Press.

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## Taurine utilization by Purple non-sulfur bacteria

Paula Suarez-Sanchez. Marine Biological Laboratory.  
Microbial Diversity Course. 06/10-07/27, 1995.

### Introduction

Taurine (2-aminoethanesulfonate) is an amino acid analogue of  $\beta$ -alanine and occurs in nature as a result of cysteine catabolism. In animal tissues and in urinary excretions taurine is present in both free and bound forms. Despite its presence in many animal tissues, animals are unable to metabolize taurine (Shimamoto and Berk, 1979). On the other hand, taurine belongs to the sulfonate compounds (which contain a sulfur atom covalently linked to a carbon), which are widely distributed in natural habitats and are byproducts of chemical syntheses for commerce (Chien et al, 1995).

Several studies have been done on taurine metabolism and only few microorganisms have been found that can grow utilizing this compound as the sole source of carbon, nitrogen, sulfur and energy. For example, *Agrobacterium* species can use taurine as the principal source of both carbon and nitrogen, with the production of ammonia and inorganic sulfate (Stapley and Starkey, 1970). The most recent studies noted that several enteric bacteria utilized sulfonate-sulfur for respiratory but not fermentative growth, perhaps reflecting the need for molecular oxygen in the attack C - S linkage (Uria-Nickelsen et al, 1993). In order to assess this possibility, purple non sulfur bacteria present an interesting type of microorganisms since they can growth anaerobically in the light and aerobically in the dark. The main goal of this research is try to test the capacity of the purple non-sulfur bacteria to utilize taurine as sole source of sulfur as well as electron donor for phototropic growth and as sole source of carbon, energy and sulfur for respiratory growth.

### Materials and Methods

*Strain Isolation.* Bacteria were isolated from water samples of School Street Marsh using a membrane filter technique. We filtered 1.0, 5.0 and 10.0 ml samples through individual 0.45  $\mu$ m membrane filters and placed them on PNSB agar plates (see below) at room temperature, under incandescent light in anaerobic conditions (GasPak jars). After pink, purple or red colonies became visible colonies were picked and restreaked in plates with the same agar medium and also in liquid cultures using screw capped tubes completely filled. The isolates were observed by phase contrast and electron microscopy.

*Media.* All the strains were growing on PNSB medium that contains: KH<sub>2</sub>PO<sub>4</sub> 1.0 g ; MgSO<sub>4</sub> 7H<sub>2</sub>O 0.4 g ; NH<sub>4</sub>Cl 0.5 g; CaCl<sub>2</sub> 2H<sub>2</sub>O 0.05 g ;

succinic acid 1.18 g ; SL12 (trace mineral solution) 1 ml and yeast extract 0.5 g. The same medium was used for testing the ability of different sulfonates to serve as sole source of sulfur (phototrophic growth), except that  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  and the corresponding sulfonate replaced in equimolar amounts, magnesium sulfate like magnesium and sulfur source, respectively. For testing the ability of taurine to serve as carbon source and electron donor, this compound was also used in other different medium, in an equimolar amount to replace the concentration of succinic acid. In the last two medium, yeast extract was replaced by six vitamin solution to avoid other sulfur sources. Other medium without sulfonate compound was used like negative control of growth (Appendix 1). Media were adjusted to pH 6.8 and sterilized before use.

*Sulfonates utilization experiments.* Five strains growing anaerobically on PNSB agar medium were used to inoculate screw capped tubes containing the same PNSB broth, magnesium chloride plus different sulfonates broth or only magnesium chloride broth. At all, six different sulfonates was used: Taurine, MOPSO, Isethionic acid, HEPES, Cysteic acid and Methanesulfonic acid. Experiments were replicated two times for phototropic or respiratory growth, during around 3 days at room temperature. Aerobic conditions were attained by shaking flasks with 10 ml of medium on dark. Similar procedure was conducted to determine taurine like only source of carbon and electron donor

## Results

### *Strain isolation*

Five strains were isolated from water samples of School Street Marsh under anaerobic conditions but none strain become to be pure culture like was showed by light microscopy (Figures 1, 2 and 3). In all cases can be appreciated two different morphologies: non-motile rods and motile gram negative spiral shape cells. The transmission electron microscopy show cells from PNSBb and PNSB1 with a polar flagella (Figures 4 and 5).

### *Utilization of sulfonates as sulfur source*

The different isolate strains show a wide range of capacity to utilize anaerobically the sulfonates tested (Table 1). The strains PNSBa and PNSBc only couldn't grow on methanesulfonic acid mientras que PNSBb can grow in all the sulfonates, and it was the only that could used methanesulfonic acid under this condition. PNSBa, b and c, growth like the control in taurine (Figure 6) since like PNSB1 and PNSB2 couldn't grow on taurine and methanesulfonic acid. Under aerobic conditions (Table 2), all the strains grow less than the control on cysteic acid and showed a

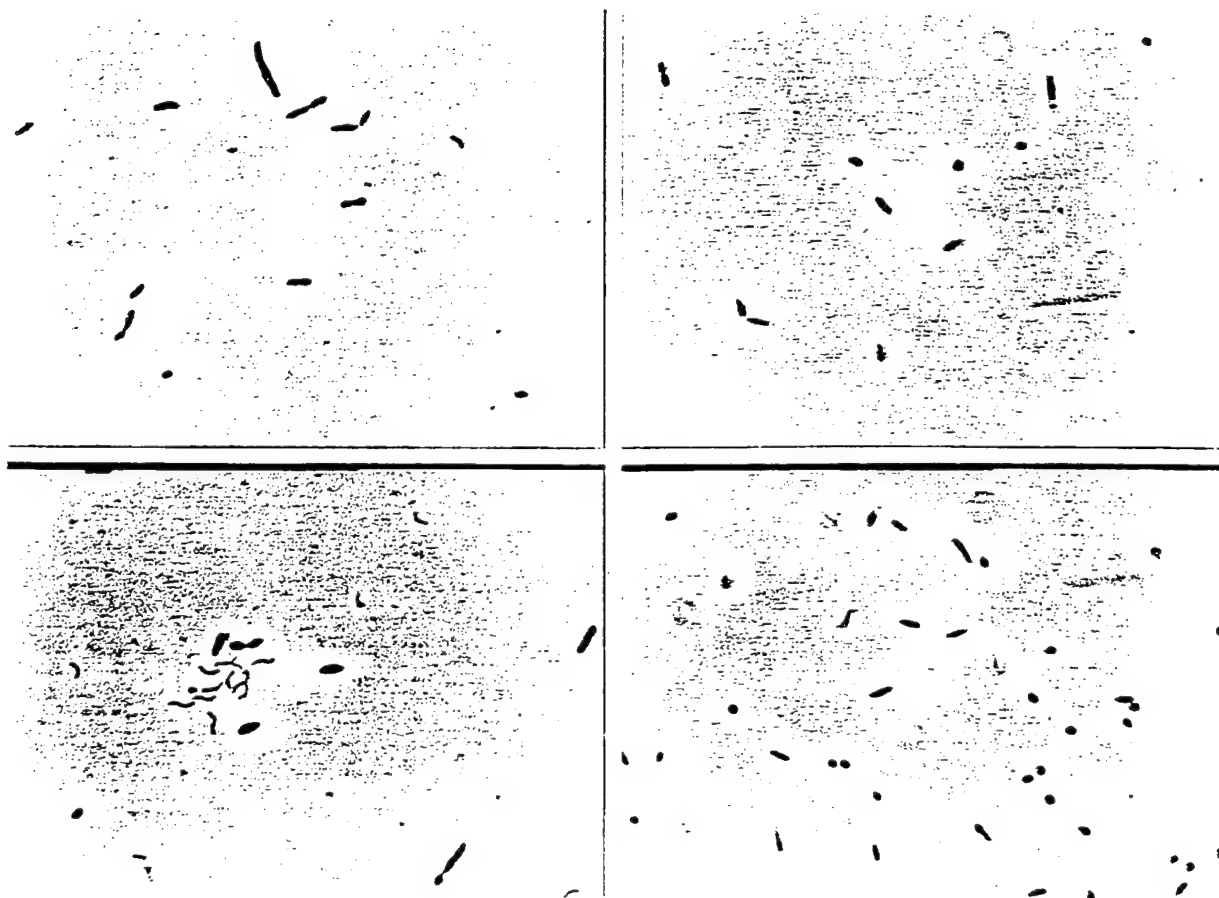


Figure 1: light microscopy of PNSBa (up) and PNSBb (down) growing on Taurine or PNSB broth (left or right, respectively).

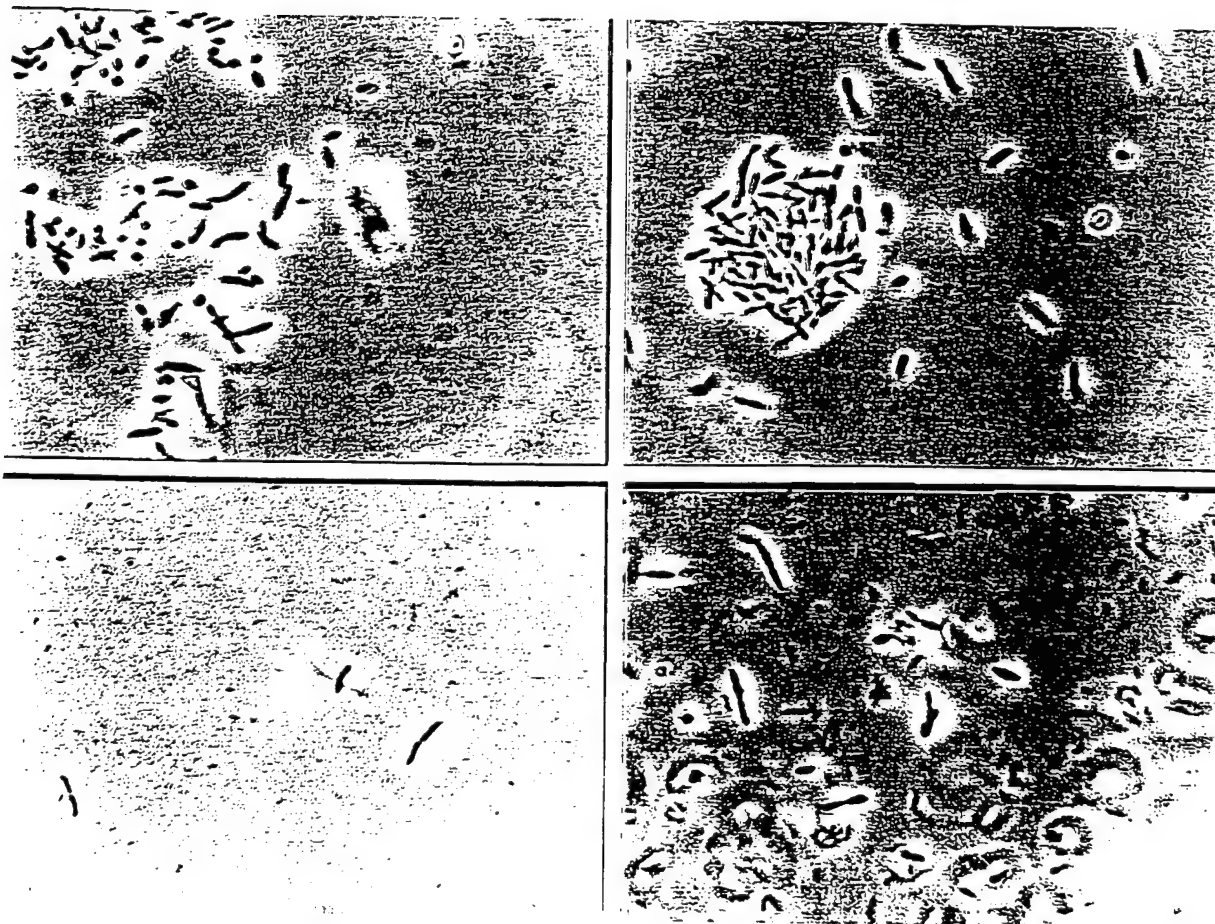


Figure 2: light microscopy of PNSBc (up) and PNSB1 (down) growing on Taurine or PNSB broth (left or right, respectively).

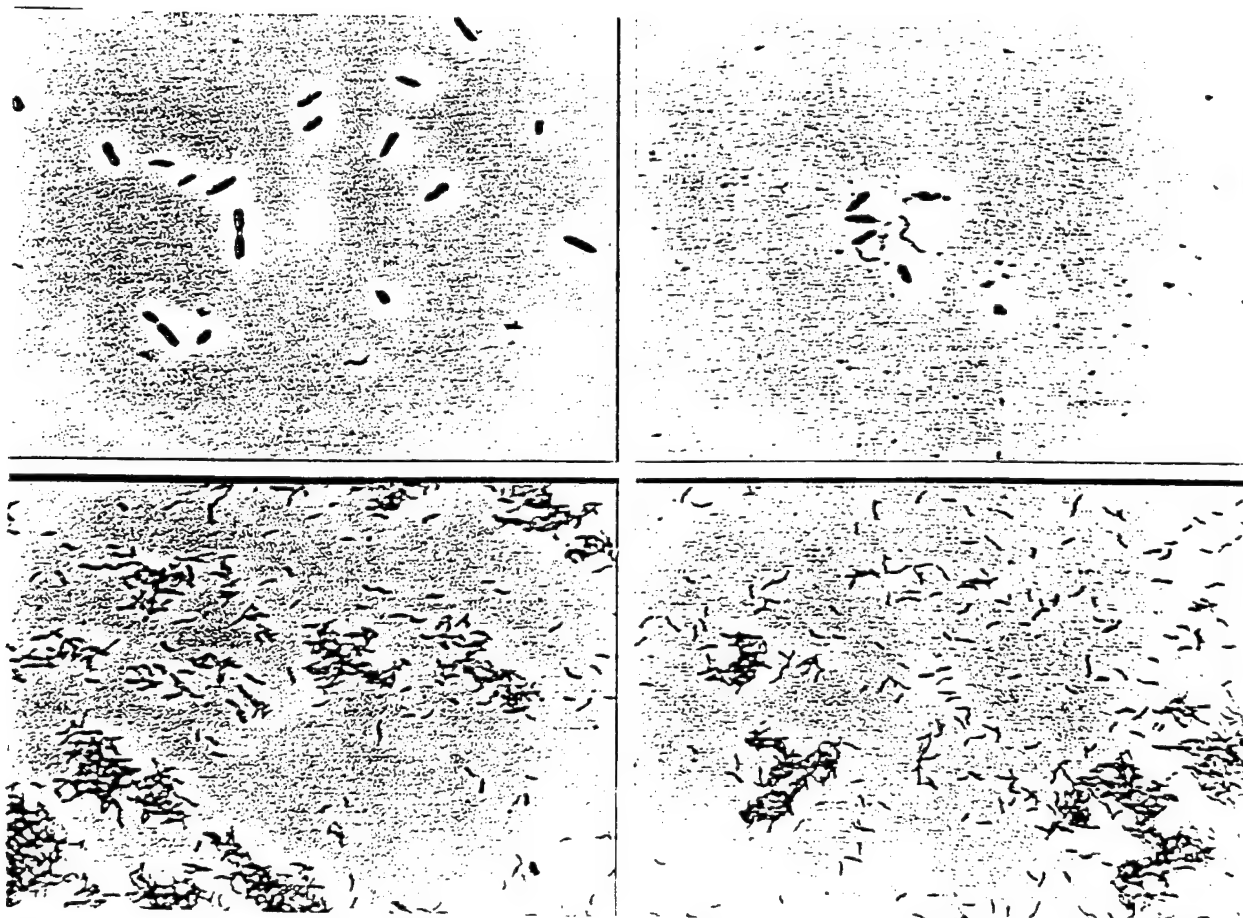


Figure 3: light microscopy of PNSB2 (up) and PNSB2 on gran stain (down) growing on Taurine or PNSB broth (left or right, respectively).



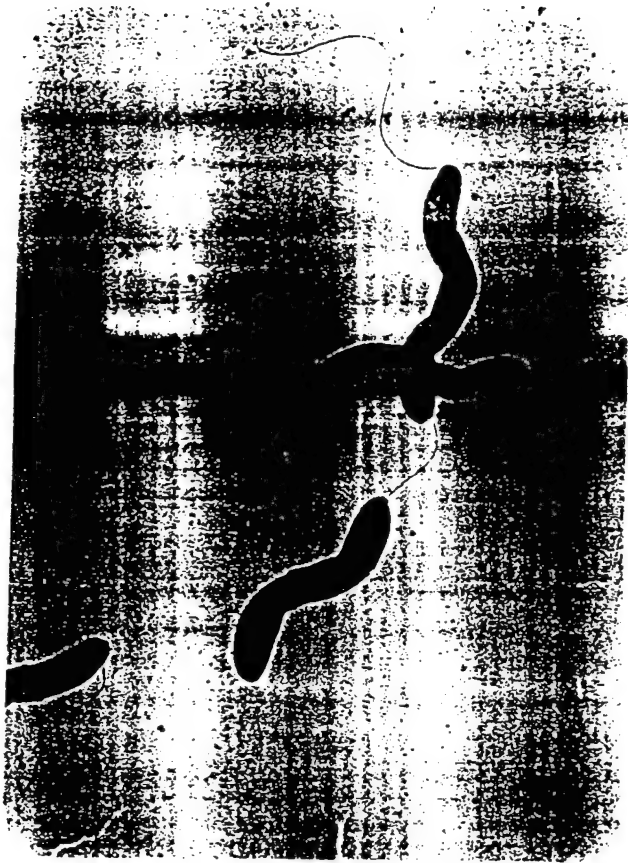


Figure 4: transmittion electron microscopy of PNSB*b* growing on PNSB broth. A polar flagella can be observed.



Figure 5: transmission electron microscopy of PNSB/ growing on PNSB broth. flagella can be observed in all the field.

PNSB strains	Taurine (e-donor)	MOPSO buffer	Isethionic acid	HEPES buffer	Cysteic acid	Methanesulfonic acid	Magnesium Sulfate	Magnesium chloride
a	-	+	+	+	d <sup>+</sup>	-	++	-
b	-	++	+	++	d <sup>+</sup>	+	++	-
c	-	+	+	+	d <sup>+</sup>	-	++	-
1	-	+	+	+	d <sup>+</sup>	-	++	-
2	-	+	+	+	-	-	++	-

Table 1: Anaerobic utilization of sulfonate compounds in some strains of purple non-sulfur bacteria (PNSB). Magnesium sulfate is the usual source of sulfate used to prepare PNSB medium (positive control). Magnesium chloride was used in the medium like negative control.

Legend:

- + ; grow
- ; no grow
- ++ ; good grow (like in positive control)
- d<sup>+</sup> ; light grow
- +<sup>+</sup> ; almost like the positive control



Figure 6: PNSBb growing on taurine broth can be observed. In the first 24 hours this strain grow better in taurine than in the positive control (PNSB broth with magnesium sulfate).

PNSB strains	Taurine	MOPSO buffer	Isethionic acid	HEPES buffer	Cysteic acid	Methanesulfonic acid	Magnesium Sulfate	Magnesium chloride
a	-	++	++	++	+	++	++	-
b	-	++	++	++	+	++	++	-
c	d <sup>+</sup>	++	++	++	+	++	++	-
1	d <sup>+</sup>	++	++	++	+	++	++	-
2	d <sup>+</sup>	++	++	++	+	++	++	-

Table 2 : Aerobic utilization of sulfonate compounds in some strains of purple non-sulfur bacteria (PNSB). Magnesium sulfate is the usual source of sulfate used to prepare PNSB medium (positive control). Magnesium chloride was used in the medium like negative control.

Legend:

++ ; grow

- ; no grow

++ ; good grow (like in positive control)

d<sup>+</sup> ; light grow

+<sup>+</sup> ; almost like the positive control

little utilization of taurine, but grow very well on the rest of sulfonates tested. MOPSO, Isethionic acid and HEPES can be used under phototropic and respiratory conditions, but cysteic acid was better used under aerobic than anaerobic conditions.

#### *Utilization of Taurine like carbon source and electron donor*

The isolated strains didn't grow when taurine was added like unique source of carbon and electron donor, don't matter the grow conditions.

#### **Discussion**

A pure culture is defined as the progeny (clone) of a single cell. To establish a pure culture, demonstrate its purity beyond doubt, and maintain it free from contaminants is one of the most important tasks for microbiologists. By careful separation of single colonies, their suspension in suitable liquid, and repeated streaking out it is possible to obtain pure cultures of the majority of microorganisms (Schlegel, 1993). Although, many times result necessary repeat this procedure a lot of times and you don't have success, like in our case. It probably happen that the bacteria grow together like a 'consortia' and is more difficult to obtain a pure culture. In our experiments, we don't take care of the fact that we don't get pure cultures and we tested the five isolate strains.

Although the strains aren't pure cultures, different sulfonates were test like sulfur source for the purple non-sulfur isolates, and was clear the utilization of MOPSO, Isethionic acid and Hepes for all the strains under aerobic and anaerobic conditions. Taurine utilization was most notorius under anaerobic (phototropic) conditions. On the other hand, the fact that methanesulfonic acid can be used as a source of sulfur by purple non-sulfur bacteria, enhance the recent finding of that enteric bacteria also can utilize this compound (Uria-Nickelsen et al, 1993). The utilization was clear only under aerobic conditions and these reveal that molecular oxygen is probably necessary to use this compound (Leadbetter, E. com. pers).

The purple non-sulfur bacteria tested weren't able to utilize taurine like sole source of sulfur, carbon and electron donor and this was also observed in *E. coli* by Uria-Nickelsen et al (1993). These researchers didn't found evidences for taurine toxicity and yet is unknown why can't be used like carbon source and electron donor. It seem that the chemical structure (carbon long-chain) of the sulfonate compounds don't affect the utilization of the compound like sulfur source, but probably does it when is used like sole source of carbon and energy. More studies about sulfonates utilization in purple non-sulfur bacteria must be done in the next future.

## Conclusions

Purple non-sulfur bacteria have great capacity to utilize sulfonates compounds, at least for what we can expect in terms of the literature.

Purple non-sulfur bacteria can use different sulfonates like sulfur source, don't matter how long is the carbon chain of the compound

More research is necessary to know more about why taurine can't be easily utilized

We thanks to Elizabeth Sherwood, Caroline Plugge, Elena Hilario-Andrade and Dianne Newman for their help in the success of this work.

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PNSB medium	Sulfonates as sulfur source	Taurine as sulfur and carbon source and e <sup>-</sup> donor
MgSO <sub>4</sub> ·7H <sub>2</sub> O	6 different sulfonates + MgCl <sub>2</sub> ·6H <sub>2</sub> O	Taurine + MgCl <sub>2</sub> ·6H <sub>2</sub> O
Succinic acid	Succinic acid	Taurine (equivalent to amount of succinic acid)
Yeast extract	Six vitamin solution	Six vitamin solution

Appendix 1: Differences on medium composition to studie utilization of sulfonate compounds like sulfur source, or Taurine like sulfur and carbon source and electron donor.



MARINE BIOLOGICAL LABORATORY  
MICROBIAL DIVERSITY 1995  
WOODS HOLE

MICROBIAL DIVERSITY IN ANOXIC MARINE SEDIMENTS

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## INTRODUCTION

In anoxic sediments, the decomposition of organic matter to inorganic nutrients,  $\text{CO}_2$  and  $\text{CH}_4$ , occurs through microbial metabolic reactions that produce increasingly less complex, dissolved, organic compounds from the sedimentary organic matter. The end products of one set of reactions are generally utilized as substrates by another set of organisms, leading eventually to the complete remineralization of the sedimentary organic matter. These processes appear to produce a limited number of substrates (*e.g.* acetate and  $\text{H}_2$ ) which are then consumed by the appropriate terminal organisms (either sulfate reducers or methanogens). After oxygen is consumed by aerobic respiration, sulfate reduction becomes the dominant form of respiration. Methane generation and accumulation becomes dominant only after sulfate in sediment pore water is depleted. Of particular interest is the near absence of methane in the high sulfate zone in marine sediments, with methane concentrations increasing in the deeper, nearly sulfate-free sediments.

The aim of this study is to describe the microbial population in coastal anoxic marine sediments, with particular emphasis on sulfate reducing bacteria (SRB) and methanogens, (the latter being the most likely representatives of the *Archaea* domain to be found in anoxic cold marine sediments) using a combined microbiological/molecular approach. The combined application of molecular techniques, which do not require selective cultivation, with physiological/ecological approaches in marine microbiology offers a means to facilitate the identification and quantitation of individual microbial species in naturally occurring microbial communities.

*In situ* hybridization with domain and group specific probes is used as a preliminary step to establish the presence of both *Bacteria* and *Archaea* in the untreated sediment samples. Selective enrichment cultivation of sulfate reducers and methanogens is combined in this study with 16S rRNA gene sequence analysis of sediment population. The combined approaches utilized in this study resulted in the description of different SRB that can be cultivated and in the identification of a number of widely diverse 16S rRNA gene sequences from *Bacteria* and *Archaea*. The comparative rRNA gene analysis of both cultured and uncultured bacterial types will place them in a phylogenetic framework, and will eventually provide information to develop a set of specific probes that could be used for future investigations of the sediment community structure.

## MATERIALS AND METHODS

*Sampling procedures.* A gravity core sample was collected in Buzzards Bay, Cape Cod, at a depth of 12m from the SSV *Corwith Cramer* (2 July 1995). As soon as the core was retrieved, the grey mud sediment was cut apart along its longitudinal axis and sub-samples were collected. The core was 75 cm long and a 3 cm thick black layer was clearly visible at a distance of about 10 cm from the top of the core. Large samples to be used as inocula for enrichment cultures were collected both from the black layer and from the bottom layer of the core and were stored in the dark. Small sub-samples from both the black and the bottom layer were quick frozen aboard the ship and kept at -20°C in the laboratory until they were processed.

*Microscopy.* Five ml of fixation buffer were added to 1g of sediment and the mix was vortexed at high setting. The mix was then put on ice for 2 min, the supernatant was discarded and the pellet was re-extracted with 2ml of fixation buffer. Cells were then fixed for 3-16h at 0-4°C. The fixation mix was centrifuged, washed twice with PBS, resuspended in 500µl of PBS plus 500µl of ethanol and stored at -20°C. Ten µl of a 100 fold dilution of cell suspension was applied to each well on the slide, allowed to dry for 20 min at 45°C and finally dehydrated for 3 min each time in 50%, 80% and 96% ethanol/H<sub>2</sub>O. Forty µl of hybridization solution containing the fluorescent labeled probe were applied to each well and incubated overnight at the estimated hybridization temperature of the probe. Slides were then rinsed with SET solution (pre-warmed at the hybridization temperature), washed 3 times for 20 min each with SET, rinsed with dH<sub>2</sub>O and finally dried. Ten µl of SET and 1µl of DAPI solution (1µg/ml) were added to each well and incubated at RT for 5-10 min, rinsed shortly with dH<sub>2</sub>O and air dried.

*Enrichment cultures.* Enrichment cultures for SRB and methanogenic bacteria were carried out in liquid saltwater media, prepared according to the Course laboratory manual. Estimation of formed sulfide in SRB cultures was performed according the Schnell procedure.

*Genomic DNA extraction.* Genomic DNA was extracted from both the black and bottom layer of the the sediment samples according to the protocol developed by Sghir and Dore (unpublished). About 100 mg of sediment were thawed on ice, resuspended in TE (Tris HCl 10mM-EDTA 1mM) and enzymatic cellular lysis was used to maximize the recovery of high molecular weight DNA amenable to PCR. No physical treatment was used in order to prevent DNA shearing. Sediment samples were incubated with lysozyme, pronase, mutanolysin and RNase at 37°C and then at 55°C after the addition of SDS and proteinase K. The polysaccharides and residual proteins were aggregated and extracted twice with an equal volume

of phenol, followed by a second double extraction with an equal volume of phenol-chloroform-isoamyl alcohol (50:49:1) and a third double extraction with an equal volume of chloroform-isoamyl alcohol (24:1) to remove the residual phenol. The genomic DNA was allowed to precipitate for 1 hour on ice after addition of 0.1 volume of 3M sodium acetate (pH 4.6) and 2 volumes of 100% cold ethanol. The DNA was then collected by centrifugation, washed in cold 70% ethanol, dried and resuspended in sterile distilled water.

*Amplification of 16S rRNA gene sequences.* The 16S rRNA gene sequences were selectively amplified from crude genomic DNA by PCR (Saiki et al., 1988) using primers designed to anneal to the conserved position in the 3' and 5' regions of 16S rRNA genes. The forward primer was 008F: 5'-AGAGTTTGATCCTGGCTCAG-3'; the reverse primer was 1517R: 5'-ACGGCTACCTTGTTACGACTT-3'. These oligonucleotides correspond to highly conserved or "universal" regions found in all known small-subunit ribosomal genes. The forward primer 344F: 5'-ACGGGGCGCAGCAGGCGCGA-3' was used together with primer 1517R to selectively amplify archaeal 16S rRNA genes. The reverse primer 385R: 5'-CGGCGTCGCTGCGTCAGG-3' was used in combination with primer 008F to selectively amplify SRB 16S rRNA gene fragments. About 50 ng of template DNA were incubated in a thermal cycler in the presence of *Taq* DNA polymerase for 30 cycles under the following conditions: 92 degrees, 2 min; 48 degrees, 30 sec; 72 degrees, 30 sec. PCR products were purified and resuspended in sterile distilled water.

*Construction of a 16S rRNA genes clone library.* Amplified 16S rRNA gene fragments were cloned into the cloning site of pCR II plasmid (Invitrogen TA cloning kit) by sticky-end ligation. The resulting ligation products were used to transform competent *E. coli* INV $\alpha$ F' cells. Clones were screened for  $\alpha$ -complementation and white colonies containing inserts were selected. Clones containing inserts of the appropriate size were identified by direct PCR screening followed by gel electrophoresis of the amplified insert.

*16S rRNA genes RFLP analysis.* Primary restriction of insert 16S rRNA gene fragments was performed by digesting the amplified 16S rRNA genes with *Sau* 3AI restriction endonuclease. The reaction products were visualized by electrophoresis on a 2.5% w/v agarose gel.

*DNA sequencing and similarity analysis.* rRNA genes from representatives of different categories of clones defined by RFLP were sequenced. Double stranded plasmid DNA templates for sequencing library clones were prepared and sequenced by standard dideoxynucleotide chain-termination methods (Sanger et al., 1977) using M13 forward fluorescent dye labeled

primer. Sequence electrophoresis was performed on a Li-Core 4000 automated sequencing apparatus. The similarity analysis was performed using the program BLASTN, which compares a given sequence to a 16S rRNA sequence database and returns a list the most similar sequences found.

## RESULTS

*Microscopy.* Subsamples of the core sediment (black layer) have been examined under the phase-contrast microscope as a preliminary approach to detect the presence of bacterial cells. No cells could be directly detected under the microscope due to the presence of particulate material in the sediment sample (Fig.1). DAPI stained bacterial cells could indeed be clearly detected in the sediment subsamples, often associated with the particulate material. Chains of cocci, sarcina-like associations, rods and vibrios were the most represented morphotypes (Fig.1). *In situ* hybridization on the same sample using a fluorescent-labeled 16S rRNA-based universal probe revealed only a minor fraction of the cells detected by DAPI staining (Data not shown). In addition to that, the few cells detected by *in situ* hybridization showed a very low level of fluorescence. Since DAPI binds to DNA while the rRNA-based probe hybridizes to the cellular 16S rRNA, this result could imply a low rRNA content in the bacterial cells. Since rRNA content is usually proportional to the growth rate, it seems likely that most of the bacterial cells detected in the subsample were not active. Actively growing cells in the enrichment for SRB at 18°C could be clearly revealed by DAPI staining and by *in-situ* hybridization using both a SRB specific fluorescent-labeled probe and a universal probe (Fig.2a).

*Enrichment cultures.* Enrichment cultures for both SRB and for methanogenic bacteria were carried out by inoculating subsamples of the black layer sediment and incubating them at three different temperatures: 18°C, 35°C and 55°C. No methanogenic bacteria were detected in any of the three conditions tested within the time schedule of the course. Enrichment cultures for SRB were carried out using either lactate, acetate or propionate as electron donors. Within four days from the inocula bacteria were actively growing in the two lactate-containing samples incubated at 35°C. No formation of sulfide could be detected in these enrichments (Tab. 1). Within four days from the inoculations, both bacterial growth and the formation of 4-10 mM H<sub>2</sub>S could be detected in the enrichment carried out at 35°C in the presence of acetate as an electron donor. The cells, mainly vibrios and curved rods, appeared to be very motile. Bacteria were actively growing in the enrichment carried out at 55°C in the presence of acetate at day 5 from the inocula. The estimated amount of formed sulfide in the culture was about 20 mM. The culture appeared to be rather homogeneous under the microscope, the predominant morphotype being represented by spore-forming rods (Fig.2b). A 0.3 ml aliquot of this enrichment culture

was transferred in bottles containing fresh medium which were then incubated both at 55°C and at 70°C. Agar shake dilutions were made to isolate single colonies, which were then inoculated in liquid medium and incubated at 55°C. Examination of one of the single colonies under the microscope revealed the presence of a pure culture of the spore-forming rods. No growth was detected either in the 55°C and 70°C transfers or in the single colony inoculum within the time schedule of the course. Ten days after the primary inoculation both bacterial growth and production of sulfide were detected in the lactate containing enrichment incubated at 35°C. The predominant morphotype in this enrichment was represented by vibrio-like motile cells.

Enrichment	Growth	Estimated H <sub>2</sub> S	Microscopy	In-situ hybrid
Lactate, 35°C/1	+	-	rods	nd
Lactate, 35°C/2	+	-	rods	nd
Acetate, 35°C	+	4-10 mM	Curved rods, cocci	-/+
Acetate, 55°C	+	~ 20 mM	Spore-forming rods	-/+
Lactate, 18°C	+	4-10 mM	Curved rods	++

Table 1: SRB enrichments.

*Estimation of molecular diversity.* Crude DNA was extracted both from the black layer and the bottom sediments and was used as a template to amplify 16S rRNA genes. Several dilutions of the crude DNA extract were used in the primary amplification to dilute eventual inhibitory substances present in the sediment. Figure 3 clearly shows that the optimal dilution for PCR amplification was  $10^{-3}$ , while higher concentrations of template DNA did not result in a detectable PCR product (Fig.3). Three sets of primers were used: universal, archaeal domain-specific and SRB group-specific, which generated respectively a 1509, 1173 and 377 bp PCR product. After cloning in pCR II plasmid vector, *E. coli* INVαF' cells were transformed with the ligation products. A total of 60 colonies were chosen after they tested positive for α-complementation of β-galactosidase, and 59 clones contained an insertion detectable by PCR. All but two of the clones contained an insert of the correct size (Fig.4). A RFLP analysis was performed on 50 of the amplification products of the correct size using Sau3AI restriction endonuclease (Fig.5). Restriction of the 50 intact 16S rRNA gene fragments with Sau 3AI

indicated that many different patterns were obtained. Seventeen clones could be grouped in 7 different RFLP patterns, while the remaining 41 clones appeared to be unique.

*Sequence similarity analysis.* Ten unique clones representative each of the three primer sets (Universal, archaeal and SRB) were selected on the basis of their different Sau3AI RFLP and were sequenced using a Li-core 4000 automated sequencer. The obtained sequences ranged from about 240 to about 500 bp in length. The sequences were then compared to a 16S rRNA sequence database and were analyzed for similarity to other known sequences. The program used in this analysis was BLASTN, which compares query sequences to the database sequences and returns a list of the most significant sequence similarities found. Four universal primer-amplified rRNA genes were sequenced and searched by BLAST: two of these sequences returned a list of different SRB16S rRNA genes as the most similar sequences found; one of them was found to be similar to two marine *Spirochaeta* 16S rRNA genes. The BLAST search for the fourth sequence returned a list of thermophilic Bacteria (mainly *Thermus* sp.), the most similar ones referring to microorganisms isolated from a Yellowstone Park freshwater hot spring. Three archaeal primer-amplified rRNA genes were sequenced. The similarity search for all of them returned a striking result: the most similar sequences found for each query belonged to the 16S rRNA genes of Crenarchaeotal sp. clones (pJP89, pJP33 and pJP41, see Fig.6) isolated from a Yellowstone hot spring environment (Barns et al. 1994). A number of other 16S rRNA genes from hyperthermophilic *Archaea*, both from marine and freshwater geothermal environments, followed in the high-scoring producing sequence list.

## DISCUSSION

Direct *in situ* hybridization on fixed sediment subsamples from the black layer with universal probes, archaeal domain-specific probes and SRB group-specific probes failed to reveal the presence of bacterial cells. In some cases two different probes, either universal or archaeal, have been used in combination in an effort to obtain a more powerful signal. Since DAPI staining of the same samples revealed, on the other hand, a considerable amount of morphologically diverse microorganisms, most of them associated with the sediment particulate material, we can conclude that in this case the sensitivity of the fluorescent-labeled probe assay is too low to reveal the presence of the cells. This is probably due to the low amount of rRNA present in the cells. On the base of this results it is possible to speculate about the relative low activity of the bacterial cells which could be detected with the DAPI staining procedure while could not be revealed by fluorescent labeling rRNA-based probes.



Enrichment cultures for SRB have been carried out using different electron donors and different incubation temperatures to select for diverse microorganisms. Different enrichments have indeed been obtained (Fig. 2a and 2b). The isolation efforts have been focussed on the 55°C enrichments, since I found intriguing the possibility to enrich for a moderate thermophilic SRB from cold marine sediments. An attempt for clearly thermophilic growth has been done incubating a transfer from the primary 55°C enrichment at 70°C, but no growth has been detected in this sample within the time schedule of the course. Due to time constraints it is not been possible to grow isolated colonies of the spore-forming rod morphotype in pure liquid cultures. Since all spore-forming SRB known to date are classified in the genus *Desulfotomaculum*, it is likely that the 55°C spore-forming SRB which have been isolated belongs to the same genus. Moreover the *Desulfotomaculum* genus contains a number of moderately thermophilic species with temperature optima of 54°C to 65°C (e.g. *D. geothermicum* and *D. nigrificans*). Since only one isolation of a still unnamed marine *Desulfotomaculum* strain has been reported to date (Keith et al., 1982), while most of the known species are from freshwater habitats, it would be interesting to furtherly characterize the 55°C growing putative *Desulfotomaculum* strain. It would certainly be very interesting to obtain the 16S rRNA gene sequence from a pure culture of this isolate, and to compare it to the collection of sequences retrieved directly from the sediment sample in order to understand whether it is represented in the collection. Furthermore, a genus or even a species-specific probe could be derived from the sequence of the pure isolate in order to probe the original sample and quantify the relative abundance of *Desulfotomaculum* species in the sediment with respect to other groups of microorganisms, although a procedure to enhance the sensitivity of the direct fluorescent labeling of cells in the sediments should be worked out.

The RFLP analysis showed a remarkable molecular diversity among the 16S rRNA genes retrieved from the sediment, both in the *Bacteria* and in the *Archaea* domain. The three Archaeal 16S rRNA gene sequences we cloned from the sediment subsample appeared to have a different Sau3AI RFLP pattern. When sequenced, the three clones showed to be indeed different, although the most striking observation was that the most similar sequences found in the database, for all the three query sequences, were a cluster of sequences from both yet uncultivated *Crenarchaeota* and isolated and well characterized hyperthermophilic *Archaea*. (Fig.6). These results are however preliminary and should be confirmed by sequencing the complete 16S rRNA genes, although the similarity score obtained in the BLAST search was very high for at least two out of three sequences. An alignment of each of the complete archaeal sequences obtained should place them in the right phylogenetic framework and indicate the closest relative to each of our clones. Although this is probably the first report of putative crenarchaeotal sequences retrieved from cold anoxic marine sediments, there have been reports



about the retrieval of both crenarchaeotal and euryarchaeotal sequences in oxygenated water off the North American coast and in Antarctica (DeLong, 1992; DeLong et al., 1994). In marine cold habitats, sediment dwelling *Archaea* usually belong to the methanogens group. Other common archaeal marine habitats are generally limited to shallow or deep-sea hydrothermal vents (methanogens and extreme thermophiles) and to highly saline land-locked seas (halophiles). The natural question that arises from the present study is: why do we find microorganisms related to the thermophiles group in a cold habitat, and how do such microorganisms make a living there?

## ACKNOWLEDGMENTS

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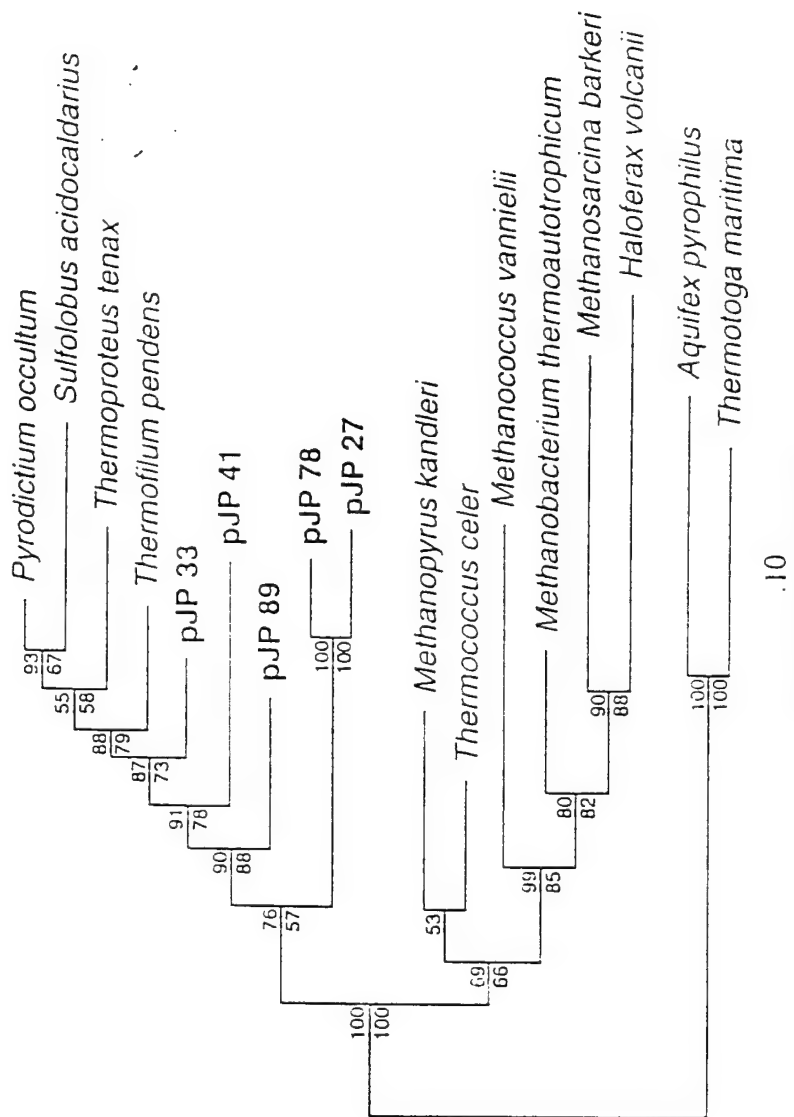


Fig 6: Archaeal phylogenetic tree (Barns et al., 1994)

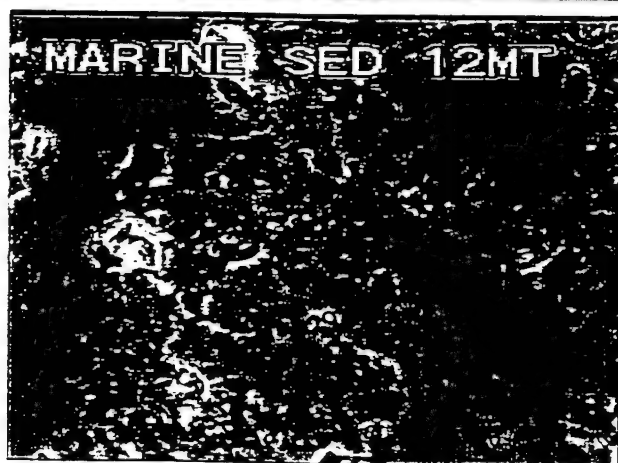
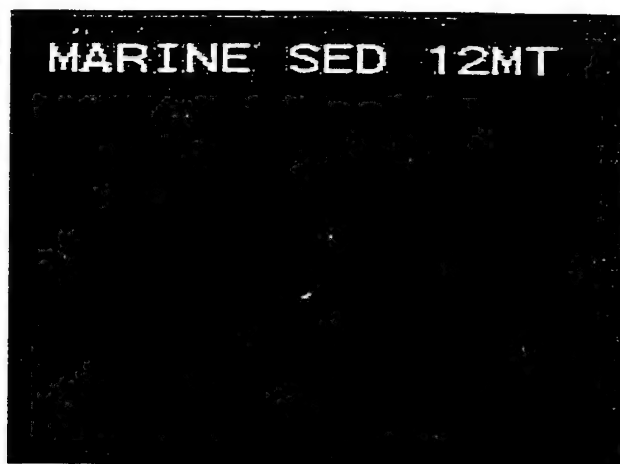
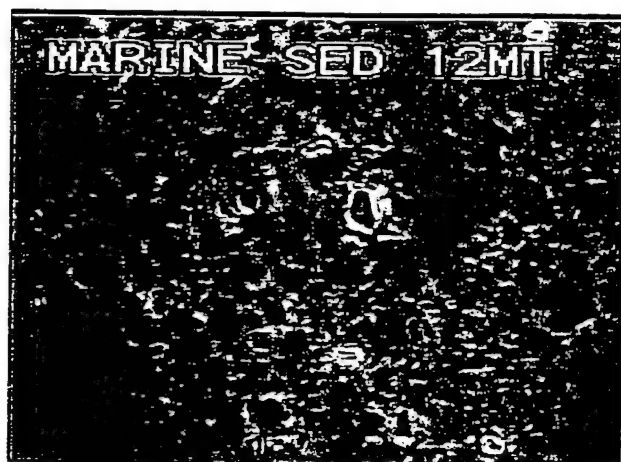
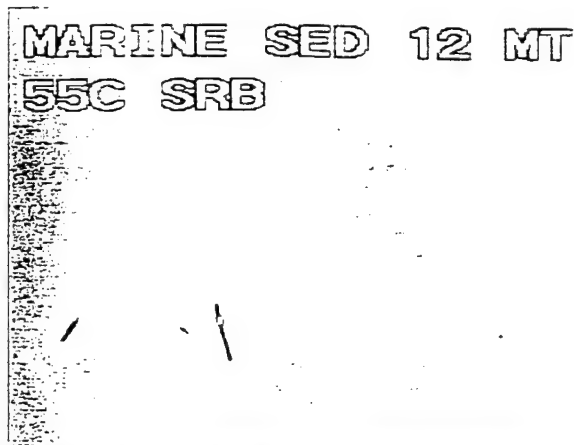
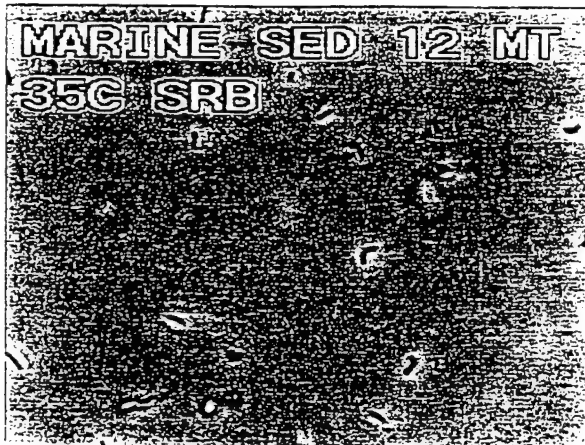
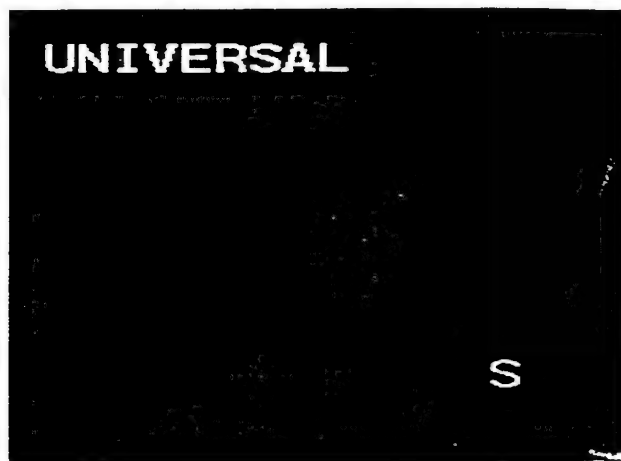
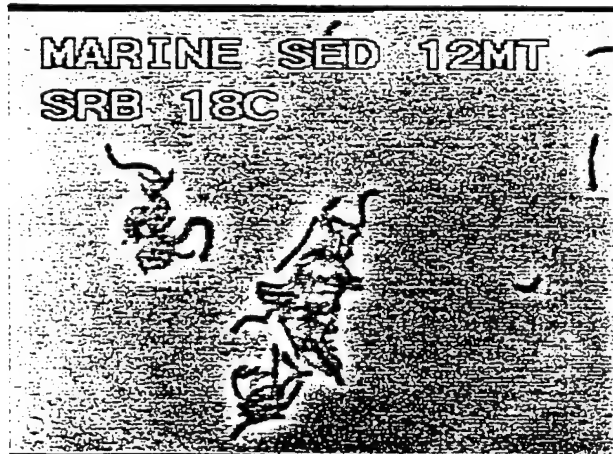


Fig 1: Phase contrast microscopy (left) and DAPI staining (right) of marine sediments



A  
Fig 2: SRB enrichments

B



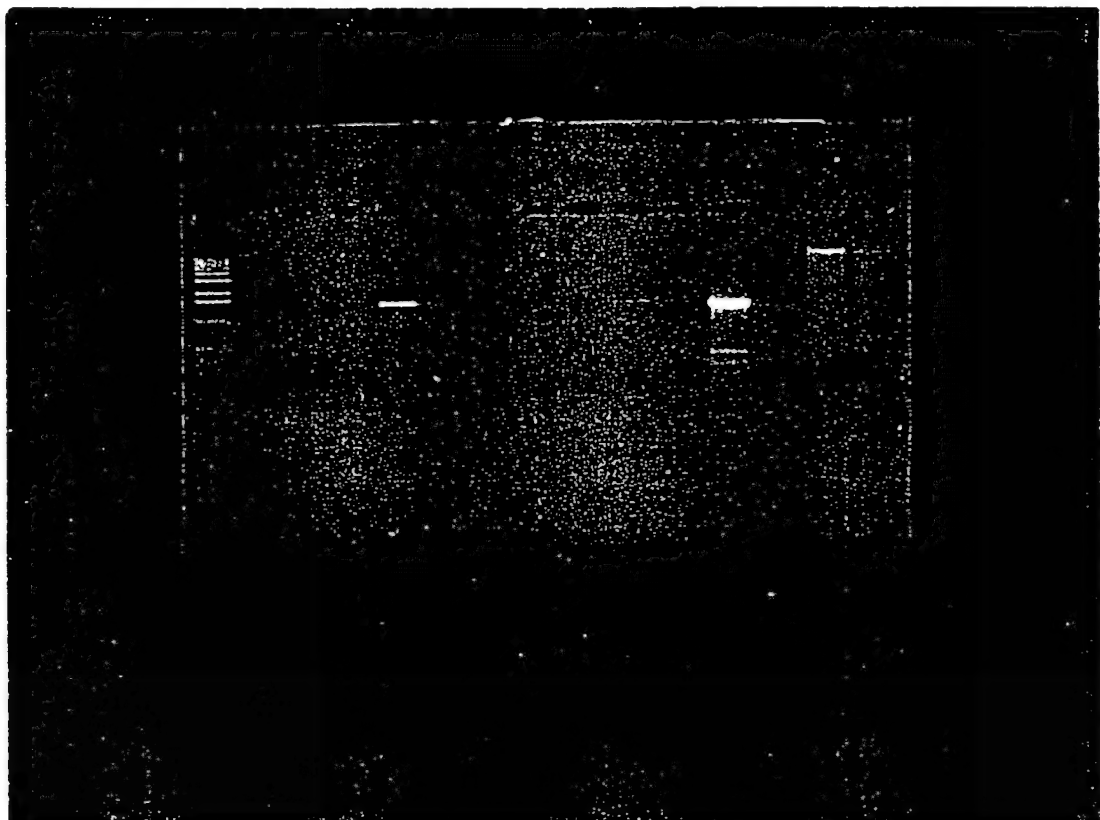


Fig 3: Primary PCR amplification of 16S rRNA from crude DNA extracts

lane 1: 1000 bp ladder

lane 2 to 6: 16S rRNA genes, black sediment. vol to  $10^{-4}$

lane 7 to 11: 16S rRNA genes, bottom sediment. vol to  $10^{-4}$

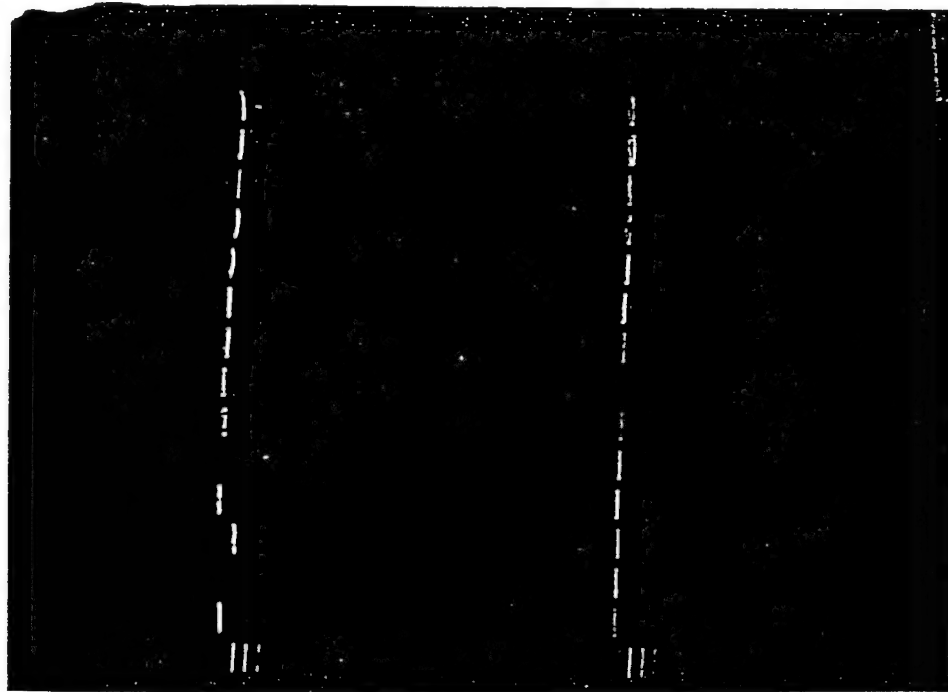
lane 12: Positive control

lane 13: Negative control

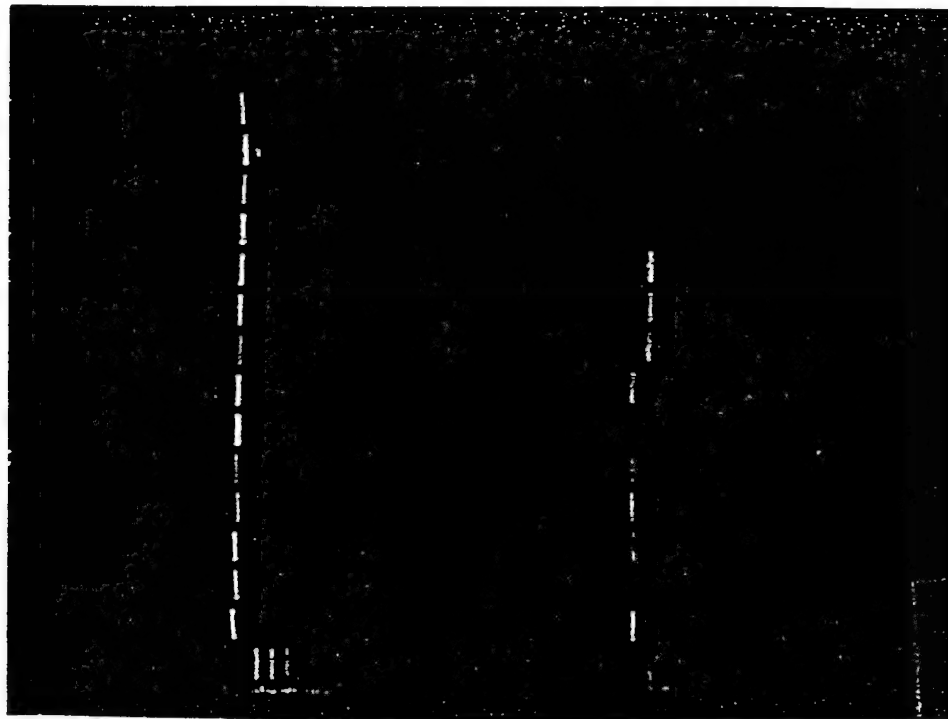
lane 14: Genomic DNA, black layer

lane 15: Genomic DNA, bottom layer

CV



A



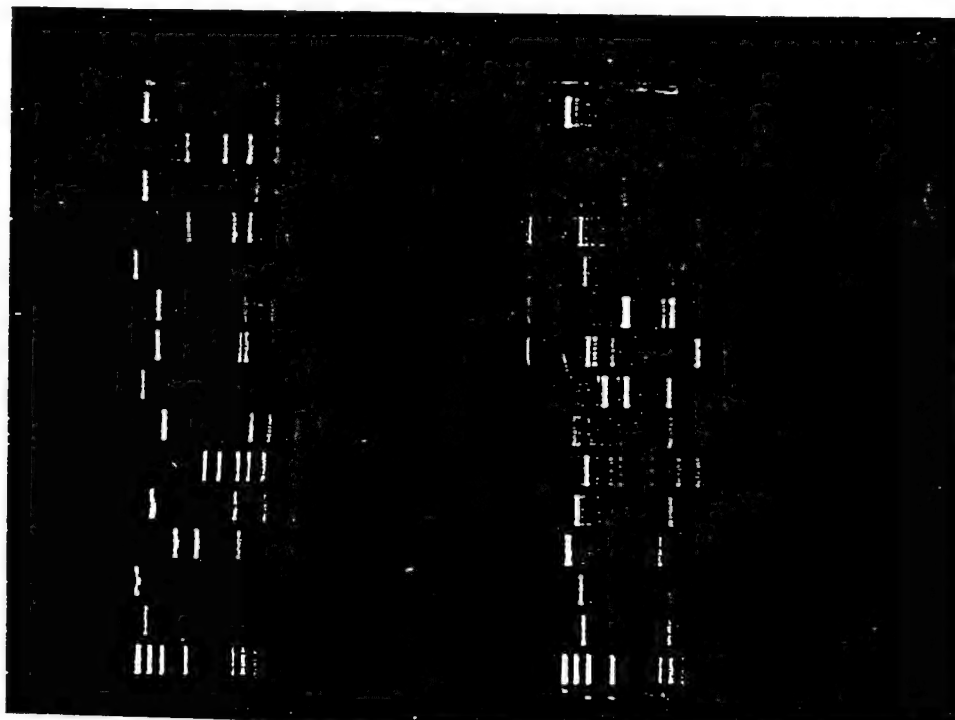
B

Fig 4: PCR amplified 16S rRNA gene fragments

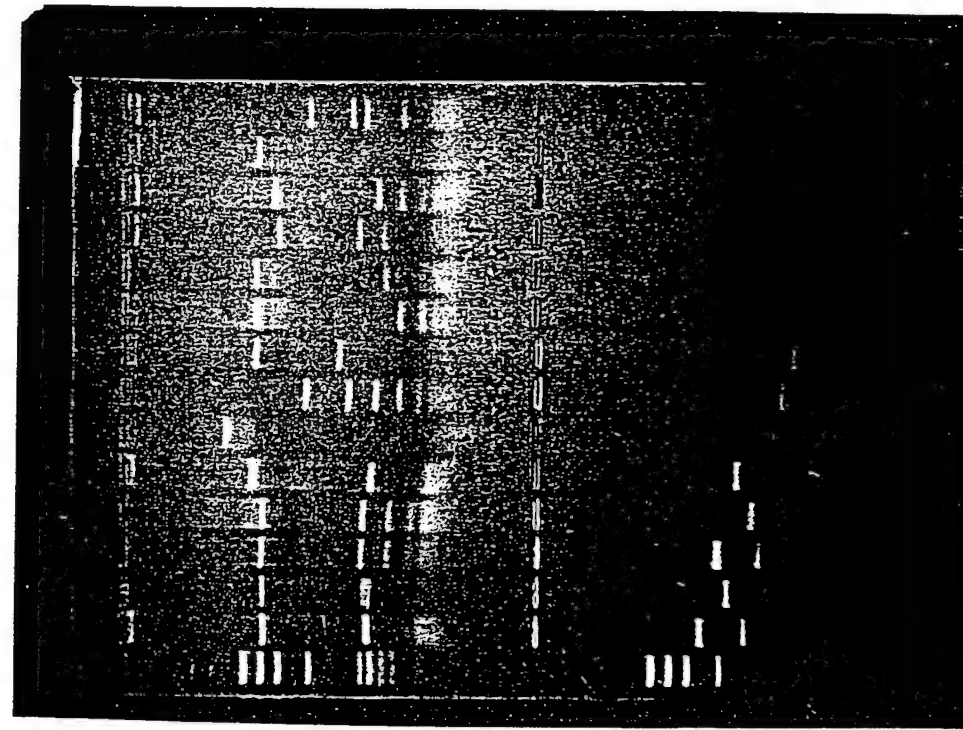
A: Universal primers

B, upper gel: Universal primers

lanes 8 to 11: Archaeal primers - lanes 12 to 15: SB primers



A



B

Fig 5: Saw 3A1 QFLP  
 A: Universal primers  
 B: lane 2 to 4: Archaeal primers

CV

## MICROBIAL DIVERSITY SUMMER CORSE 1995

Research project title:

"The effect of respiration on the phototactic behaviour of the purple nonsulfur bacterium *Rhodospirillum centenum* "

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Research done in collaboration with L.Dagmaard (Microbial Diversity course student) and C.Bauer (visiting professor during the Microbial Diversity course)

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### INTRODUCTION

In a recent review on phototaxis and other sensory phenomena in purple photosynthetic bacteria, Howard Gest (1) reports and interesting consideration by Presti and Delbruk (2) about the use of light by living organisms: " as a source of energy and as a means of obtaining information about their environment". Photosynthetic organisms would therefore be expected to use light for sensory functions as well as for energy-requiring processes, and many are known to exhibit the phenomenon of phototaxis, defined as the oriented movement of a locomotive organisms with respect to the *direction* of light. The movement can either toward a light source (positive phototaxis) or away from the source (negative phototaxis).

It has long been proposed that phototaxis provides the photosynthetic organisms with the capability of orienting itself at light intensities optimal for growth. However, the fact that phototaxis has been observed also in non-photosynthetic organisms, e.g. *Isosphaera pallida* (3), clearly indicates that this phenomenon is *per se* independent of "photosynthesis". It is evident that the energy required for light-induced movements can be supplied in various ways, namely: by photosynthetic electron transport, by respiration and, possibly, by fermentation.

Phototaxis has been studied and unambiguously documented in algae and cyanobacteria (2); surprisingly, in over a century of research on this topic, the phototactic behaviour of a nonsulfur purple bacterium, *Rhodospirillum centenum*, has clearly been demonstrated only in 1994 (4). Indeed, "swarm agar-colonies" of *Rsp.centenum* are capable of macroscopically visible phototactic behaviour. This is a remarkable and unique feature of this bacterial species and it is paralleled by a production of numerous lateral flagella (5).

In this study we analyze the effect of respiration on the phototactic behaviour of *Rsp.centenum*. To this purpose we have been using both biochemical and genetic approaches, namely: a) detection of the respiratory capability of "single agar-



colonies" by means of a oxygen microelectrode (see Revsbech 1986) under variable respiration-inhibited conditions either in the light or in the dark; b) correlation of the respiratory activities by isolated membranes, with the "photomovement" of the agar-colonies c) isolation of mutants altered in respiratory electron transport obtained by Tn5 transposon mutagenesis with an IncP plasmid vector as described by Yildiz et al. (1991).

## MATERIALS AND METHODS

### 1. Bacterial strains, media and routine growth conditions.

The bacterial strains used in this study were *Escherichia coli* SM10/ Lpir/ pTn5-spR and *Rhodospirillum centenum* SW (ATCC 51521) and YB707 (bacteriochlorophyll deficient mutant). *E. coli* cells were grown at 37°C in Luria broth (7). For liquid dark-aerobic growth, cells of *Rsp. centenum* were cultivated at 42°C in either 30ml or 1 liter flasks using PYVS (0.3% peptone, 0.3% yeast extract, 0.4% soytone, 20ng/ml vitamin B12 and 1.5 µg/ml biotin) medium as described previously (5).

### 2. Membrane isolation procedure, respiratory assays.

Cells of *Rsp. centenum* SW and YB707 were grown in CENMED medium as previously described (5) and harvested at late log phase (0.8-0.9 A at 660nm) and cells resuspended in MOPS (pH7.4), MgCl<sub>2</sub> 10mM buffer at approx 0.1g (wet wt)/ml. Membrane fragments were prepared by following the French-pressure cell method (see 8).

Respiratory activities were determined by optical spectroscopy as described previously (8).

### 3. Mutagenesis.

Transposon mutagenesis was accomplished by using a transposon delivery system of *E. coli* involving mobilizable IncP plasmids delivering resistance to spectinomycin; as reported, *Rsp. centenum* is naturally resistant to high levels of kanamycin. Coniugal transfer of the IncP plasmid was performed by mixing appropriate aliquots of donor and recipient (8:1 and 10:1 ratios) and then concentrating them by filtration through a 0.45 µm-size-pore filter (Gelman GN-6). The filter was placed onto PYVB agar medium, incubated at 37 C for 24 h and then washed with 3ml of PYVB medium. 0.2 ml of the resuspended cells were then plated onto selective plates (PYVS + Km 50µg/ml + Sp7.5µg/ml + 1.5% agar). The plates were incubated at 37°C for 60 h to obtain isolated colonies. The colonies were subsequently streaked out on CENMED agar plates (+Km 50µg/ml + Sp 7.5µg/ml) to further reduce the *E. coli*-background. Respiratory deficient mutants were selected through the use of the NADI-oxidase test (alfa-naphthol+DMPD) which specifically identify colonies defient in cyt c oxidase activity

#### 4. Oxygen microelectrode technique.

Oxygen consumption by single swarm colonies was measured using a Clark-microelectrode (diameter of the tip approx 12 $\mu$ m) by essentially following the procedure described by Revsbech (9). This electrode is insensitive to stirring and allows to determine oxygen variations within a 1/2time of 0.4sec.

## RESULTS AND DISCUSSION

The photosynthetic purple nonsulfur bacterium *Rsp.centenum* can grow either in illuminated anaerobic environment by using photosynthetic electron transport to generate an electrochemical proton gradient or heterotrophically in dark aerobic conditions by using respiratory electron transport. In contrast to other purple non sulfur species, e.g. *Rb.capsulatus* and *Rsp.rubrum*, in which the synthesis of the photochemical apparatus is controlled by the oxygen partial pressure (see Zannoni 1995) *Rsp.centenum* synthesizes a functional photosystem under both aerobic and anaerobic conditions (Yildiz et al. 1991). This peculiarity allows to observe light-driven colony mobility (phototaxis) under both aerobic and anaerobic conditions. For simplicity, we conducted all of the mobility experiments shown here, under aerobic conditions at 37-40°C (light intensity of 3 $\mu$ E m<sup>-2</sup> sec<sup>-1</sup>).

It has previously been shown that in a low light intensity (approx 5  $\mu$ E) the so called "phototactic" response of *Rb.sphaeroides* cells is negatively affected by oxygen (Armitage et al. 1985). Although the phototactic behaviour of this bacterial species can more properly be described as a "scotophobic" effect since the cells are moving toward a gradient of light intensity, it is apparent that under non-saturating light intensities ( $\ll$ 50  $\mu$ E) oxygen affects the cell movement. This conclusion is in line with an early study (Rugolo and Zannoni 1983) on intact cells of semiaerobically dark-grown *Rb.capsulatus*, demonstrating that the light-generated membrane potential is inhibited by respiratory electron transport. The rationale for this result is that photosynthetic and respiratory apparatuses are intermingled in such a way they share a series of redox components, i.e. soluble cyt c and ubiquinone molecules.

The results obtained in both *Rb.capsulatus* and *Rb.sphaeroides* predict that if the phototactic response of *Rsp.centenum* strictly depends upon the electrochemical proton gradient generated by light, inhibition of respiration would enhance the locomotive capacity of this bacterial species.

#### 1. The redox chain of aerobically dark-grown *Rsp.centenum*.

##### 1.1 Spectral analysis.

Figure 1 shows the reduced-minus-oxidized difference spectra of both isolated membranes and the 140.000xg supernatant from dark-grown cells of *Rsp.centenum*. It is apparent that the "soluble" fraction contains a consistent amount of cytochrome(s) c (peak at 552 nm and a shoulder at 550.5 nm) while the membrane fraction shows the presence of both c- (peaks at 552 and 556nm) and b-

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type haems (peaks at 558 and 560nm). Using extinction coefficients of  $19\text{mM}^{-1}$  and  $22\text{mM}^{-1}$  for cyt *c* and *b*, respectively, we calculated that also isolated membranes contains high amounts of *c*- and *b*-cytochromes (2.9 and 1.8 nmol mg prot<sup>-1</sup>, respectively); notably, the amount of *c*-type haem released in the 140.000xg supernatant during the cell-fractionation by mechanical treatment was equal to the one present in intact membranes (see Materials and Methods). The latter finding strongly suggests that intact cells of *Rsp.centenum* are endowed with a periplasmically-located cytochrome(s) *c* which is likely to be involved in connecting the redox-chain to the photosynthetic reaction center and/or to a high-potential membrane-bound oxidase. This conclusion is supported by evidence that addition of Na-ascorbate ( $E_m = +65\text{mV}$ ), which poises the redox-potential of an open-cuvette sample at approx +150mV, reduces 1/3 of the membrane-bound cyt *b* and 90% of the soluble *c*-type cytochrome(s) (see trace c).

## 1.2 Respiratory activities

Table 1 shows a series of respiratory activities measured in isolated membranes from aerobically dark-grown *Rsp.centenum*.

Table 1.

Electron donors	Inhibitors	Electron acceptors	Activities (*)	% of the control
NADH	/	oxygen	1.60	100
NADH	rotenone	oxygen	0.12	8
NADH	antimycin A	oxygen	0.47	29
NADH	myxothiazol	oxygen	0.32	20
NADH	/	cyt c <sup>(a)</sup>	3.03	100
NADH	rotenone	cyt c <sup>(a)</sup>	0.59	19
NADH	antimycin A	cyt c <sup>(a)</sup>	1.57	52
NADH	myxothiazol	cyt c <sup>(a)</sup>	0.60	20
Cit C <sup>(b)</sup>	/	oxygen	25.80	100

(\*) Activities are expressed as  $\mu$  moles of either electron donor or acceptor h<sup>-1</sup> mg of protein<sup>-1</sup>

(a) Measured in the presence of 5mM cyanide to avoid cyt *c* oxidation after reduction by NADH.

(b) Horse-heart cytochrome *c* was 80% reduced by addition of Na-ascorbate.

Additions and non-standard abbreviations: NADH, 0.1mM; cyt *c* (horse-heart cytochrome *c* SIGMA) 0.1mM; rotenone and myxothiazol, 1 $\mu$ M each; antimycin A, 3 $\mu$ M.

The data of Table 1 clearly show that membranes from dark-grown *Rsp.centenum* present a NADH oxidase activity which is fully sensitive to rotenone (1 $\mu$ M) and partially sensitive to antimycin A and myxothiazol, two specific inhibitors of complex III. This suggests that the redox chain is endowed with a ND-1 type dehydrogenase delivering reducing equivalents to an orthodox bc1-redox complex (Gennis et al. 1993). On the other hand, the presence of a significant residual respiratory activity insensitive to both antibiotics is indicative for the presence of a branched chain in which one of the two branches is formed by a membrane bound quinol oxidase (see Zannoni 1995).

In Fig.2, the patterns of the NADH and cyt c oxidation activities as a function of increasing concentrations of cyanide, are shown. It is evident that oxidation of exogenously added reduced-cyt c is completely inhibited by 50 $\mu$ M cyanide, a concentration which considerably reduces (80%) also the NADH consumption, the remaining 20% of activity being inhibited only by 5-10mM cyanide. Notably, the respiratory inhibitor NaN<sub>3</sub> is far less efficient in inhibiting the total respiratory activity of *Rsp.centenum* (75% inhibition at 1-2mM) being however more specific in blocking the cyt c oxidase activity although used at mM concentrations.

## 2. Phototactic response of *Rsp.centenum*.

The experiments to test the swarming properties of *Rsp.centenum* (reported in Table 2) were performed as following: cells (1ml harvested at 0.9 A) were concentrated and resuspended in 40 $\mu$ l of PYVS medium. For inhibition experiments, the inhibitors were incubated approx 5 min at room temperature before centrifugation. The concentrated cells (40 $\mu$ l) were placed onto side of PYVS squared-plates and let the spot dry for a few minutes at room temperature. The plates were subsequently incubated in the dark at 42°C for 4 h before subjecting them to light source (3 $\mu$ E m<sup>-1</sup> sec<sup>-1</sup>) at approx 37-40°C for 3 h. As previously described, the dark incubation period is required to induce formation of lateral flagella (Ragatz et al. 1995). The approx swarming rates of wild type cells were 10-15 mm/h on 0.8% agar-PYVS plates. Occasionally we observed swarming rates of approx 20 mm/h.

The results summarized in Table 2, rows 2 and 5, indicate that the swarming capability of *Rsp.centenum* is strongly repressed by those inhibitors affecting the photosynthetic electron transport such as antimycin A and myxothiazol. Conversely, Na-azide which inhibits the cyt c oxidase (see Table 1) significantly stimulates (120%) the swarming capacity of *Rsp.centenum*. This indicates that in the light the oxygen consumption by the respiratory electron transport reduces the swarming rate of this bacterial species so to suggest that respiration plays a minor role in phototaxis. This is confirmed by the data obtained with YB707 cells, a mutant which is proficient in aerobic growth but it has also no phototactic response (Tab. 2); thus, the light-dependent electron flow is required for locomotion of

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*Rsp.centenum*. In this respect, the effect of rotenone (20% inhibition) is interesting because rotenone is not expected to affect photosynthetic electron transport unless to consider that it might also inhibit the light-dependent formation of NADH (by reversal electron flow) which is presumably required for the cell movement. It is noteworthy that addition of asc/DCIP slightly reduces the swarming rate of the cells; this is consistent with the fact that reduced DCIP is likely to stimulate respiration (see below, paragraph 3).

Table 2.

Swarming rates of *Rsp.centenum* w.t. and YB707 strains on 0.8% agar-PYVB plates. The rates are expressed as mm/h

Additions (concentrations)	STRAINS		
	W.T.	% of the control	YB707
1. ----	14.0	100	0.0
2. antimycin A (5 $\mu$ M)	7.4	53	/
3. Na N <sub>3</sub> (10mM)	16.8	120	/
4. Rotenone (10 $\mu$ M)	11.2	80	/
5. Myxothiazol (5 $\mu$ M)	0.0	0	/
6. CCCP (10 $\mu$ M)	0.0	0	/
7. Myxo+ Asc/DCIP	0.1	0	/
8. Asc/DCIP	10.1	72	/

The data are the average of two independent sets of experiments

### 3. Effect of light on respiration of swarming colonies on agar-plates as detected by a oxygen microelectrode.

Changes in oxygen concentration occurring within a swarm colony on agar plates during light-dark cycles can be analyzed using a oxygen microelectrode (see Revsbech and Ward 1983). A series of oxygen profiles obtained by quickly advancing the electrode (tip size of 12 $\mu$ m, 80% response time 0.5sec) from the colony surface and downward, stopping for a 3s period at 25 $\mu$ m intervals, was performed. The average depth of the colonies was approx 100 $\mu$ m and the electrode was fixed at 50-70 $\mu$ m depth corresponding to a oxygen concentration of 80-130 $\mu$ M. As previously described (see Rev. by Revsbech and Jorgensen 1986) the slope of the oxygen profile results from the difference between the rate of the oxygen diffusion and the rate of oxygen consumption by the cells on condition that the depth of the agar-colonies does not vary. The oxygen profile of myxothiazol treated cells was considerably different (decrease of the slope-profile of 38%) from the control while a

slight, but significant increase (120%) of the slope-profile was seen in the presence of the protonophore CCCP and of the redox couple ascorbate/DCIP (not shown). Figure 3 shows the oxygen pattern by the swarming colonies when subjected to short periods (30 sec) of IR light ( $0.3 \mu\text{E m}^{-2} \text{sec}^{-1}$ ). As expected, light has a strong inhibitory effect on respiration by the swarming colony resulting in a rapid increase of the oxygen concentration (from 95 to  $148 \mu\text{M}$ ) around the microelectrode tip (approx  $10 \mu\text{m}$  radius). Conversely, in myxothiazol treated colonies (no phototactic response, see Tab.2), the light-period seems to stimulate the respiratory rate so that the oxygen concentration drops from 81 to  $56 \mu\text{M}$ ; this result was confirmed by the pattern shown in trace (d) in which the oxygen concentration of myxothiazol treated cells is strongly reduced by light in the presence of ascorbate/DCIP (from 110 to  $64 \mu\text{M}$ ). Conversely, when the cells were treated with the uncoupler CCCP (no phototaxis, see Tab.2) the respiratory activity of the colony was still inhibited by light leading to an increase in oxygen concentration (trace c, from 75 to  $114 \mu\text{M}$ ). This latter finding is clearly in contrast with the proposal that inhibition of respiration by light is due to a thermodynamic control upon the rate of oxygen consumption (Cotton et al. 1983) but instead it supports the idea that in some circumstances reducing equivalents may switch between photosynthetic and respiratory components (Zannoni et al. 1978; Rugolo and Zannoni 1983).

#### 4 . Transposon mutagenesis

The transposon mutagenesis protocol described under Materials and Methods led to isolate a series of colonies carrying both kanamycin and spectinomycin resistance. However, the low frequency (5-6 pigmented colonies per plate) and the strong background on PYE plates suggested to select for those colonies able to grow on CENMED agar (Km50+Sp7.5) plates in the dark. A screening for NADH-negative (pigmented colonies) was performed and two mutants that presented pink and brown colonies, respectively, were streaked out on CENMED-agar plates and let them incubate at  $37^\circ\text{C}$  for 6 days. After this period we obtained two different types of colonies, both NADH-minus, of approx 1mm (brown) and 0.3mm (pink) diameter with a very poor background. No further characterization of these mutants has been performed (the Microbial Diversity summer course was over!!).

#### CONCLUSIONS

An important conclusion of this work is that the positive phototactic behaviour of *Rsp.centenum* is negatively affected by respiration. This result, obtained through the use of the oxygen microelectrode technique (Table 2 and Fig.3) strongly suggests that photosynthetic electron flow is essential for cells movement as also demonstrated by the lack of phototaxis of the bacteriochlorophyll deficient mutant YB707 which is however proficient in dark-growth. We have also been able to demonstrate that the facultative phototroph *Rps.centenum* synthesizes a branched respiratory chain when grown aerobically in the dark. A variety of different branched respiratory chains has been found in both facultative phototrophs, such as for examples *Rb.capsulatus* and *Rsp.rubrum*, and aerobes (see

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Zannoni 1995). The high-amount of membrane-bound c-type cytochromes (Fig.1) found in *Rsp.centenum* suggests the presence of a c-type tetrahaem/reaction center; this type of reaction center resembles that of *Rps.viridis* in which the role of the periplasmically located loosely bound cyt c is however still a matter for debate due to its low concentration. Thus, *Rsp.centenum* could be the first facultative phototroph in which the interaction between soluble-c and the c-tetrahaem/RC could hopefully be demonstrated easily due to the presence of high-concentrations of soluble c. Another aspect to be further investigated is the effect of the oxygen tension on the synthesis of cytochromes since we know that, in contrast to other facultative phototrophs, the pigment synthesis by *Rsp.centenum* is naturally insensitive to oxygen.

#### Acknowledgements

I would like to thank my wife (Michela) and my wonderful son (Luca) for giving me the opportunity to spend 47 exciting days at the MBL (Woods Hole) working at the bench ..... after so long time!

I want also to thank all persons (faculty staff and TAs) involved in the Microbial Diversity summer course for their advice, competence and .... patience.

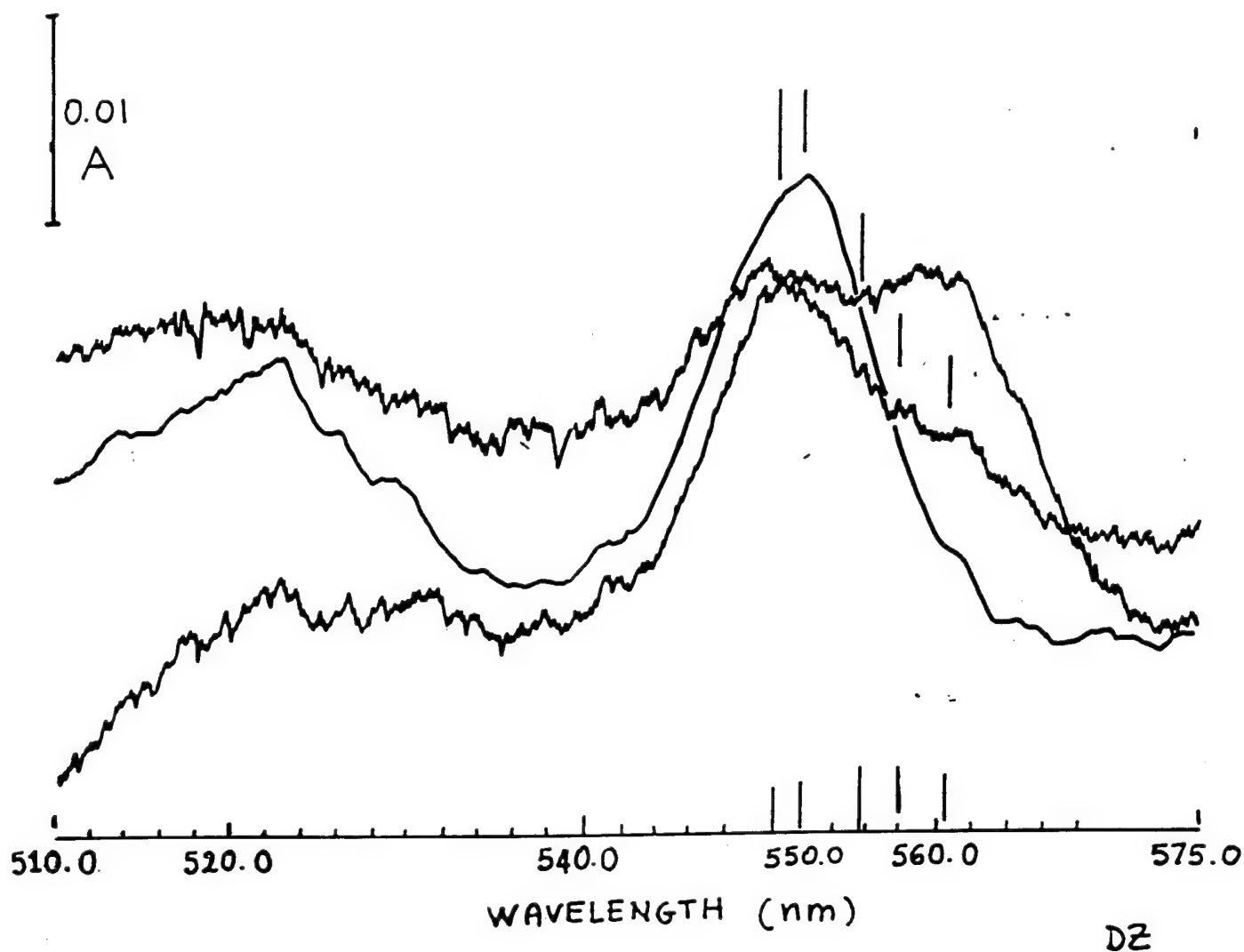
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July 24 1995..... "the game is over, no more time to play!"



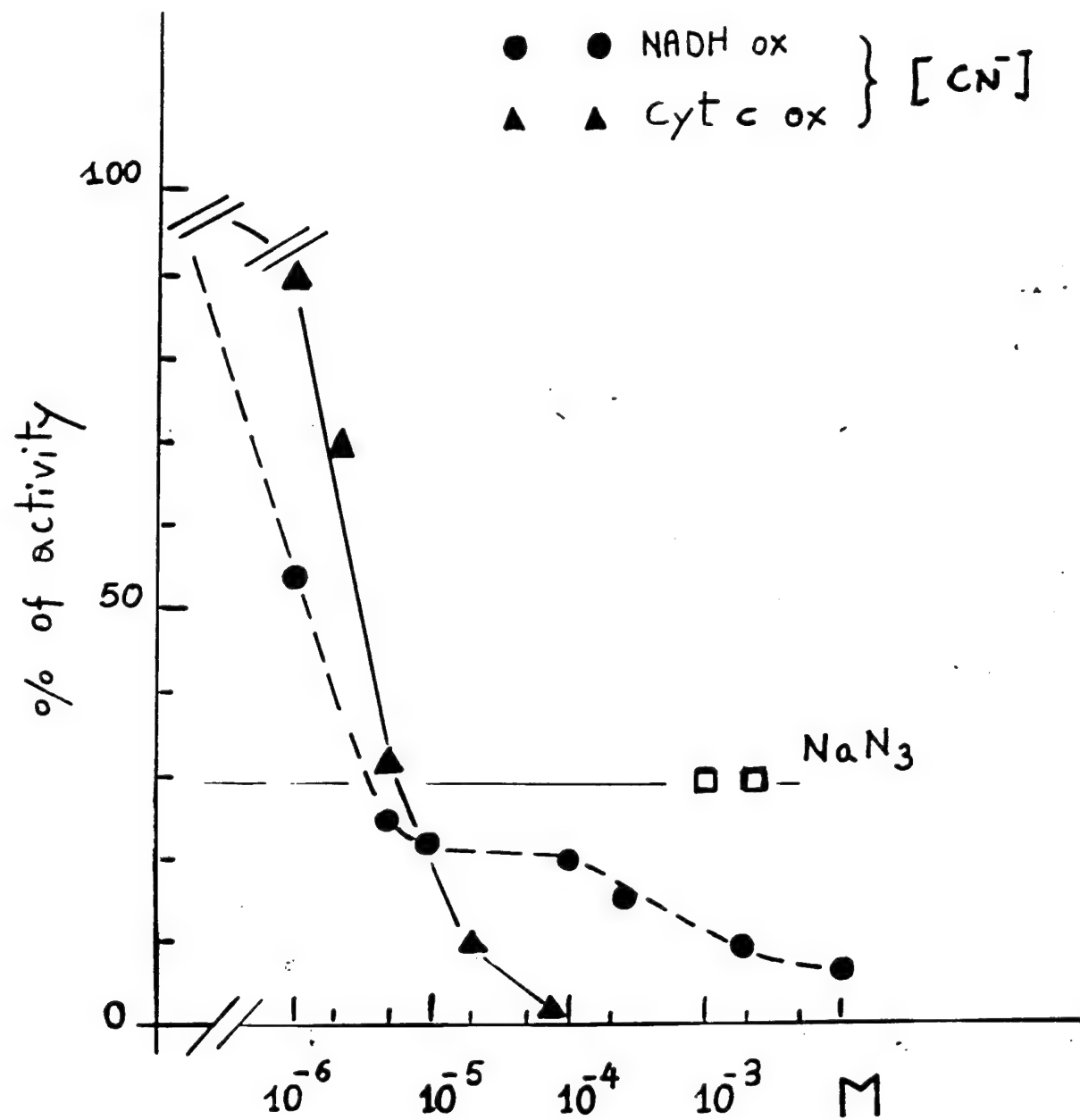
Fig. 1



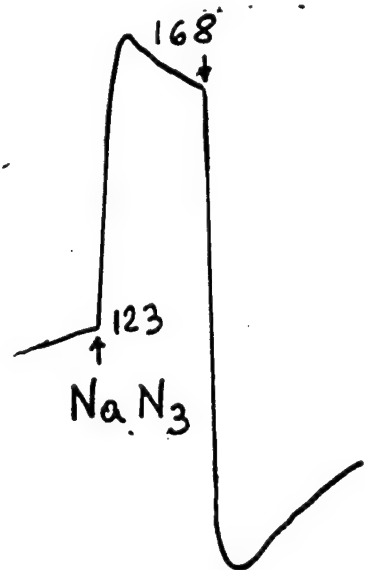
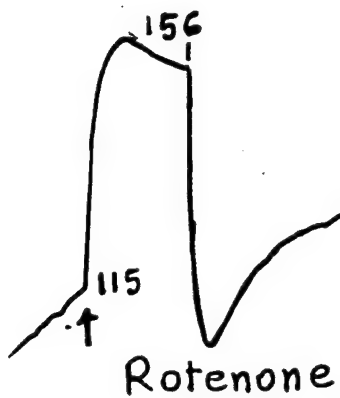
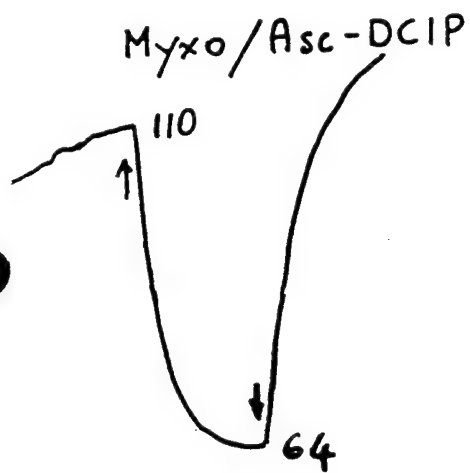
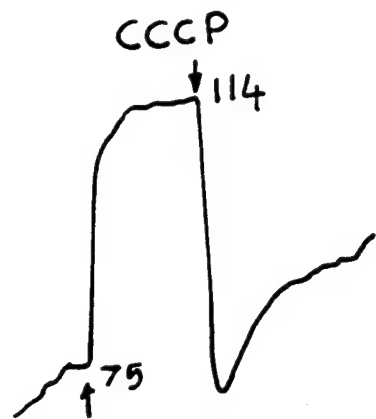
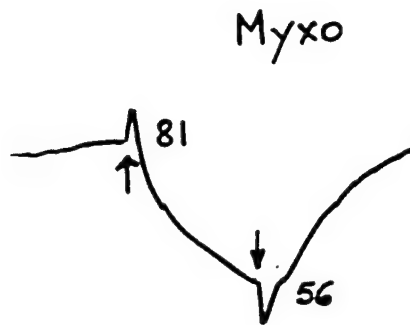
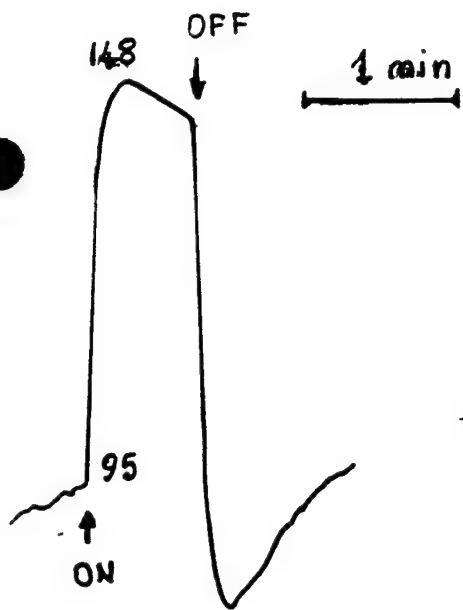
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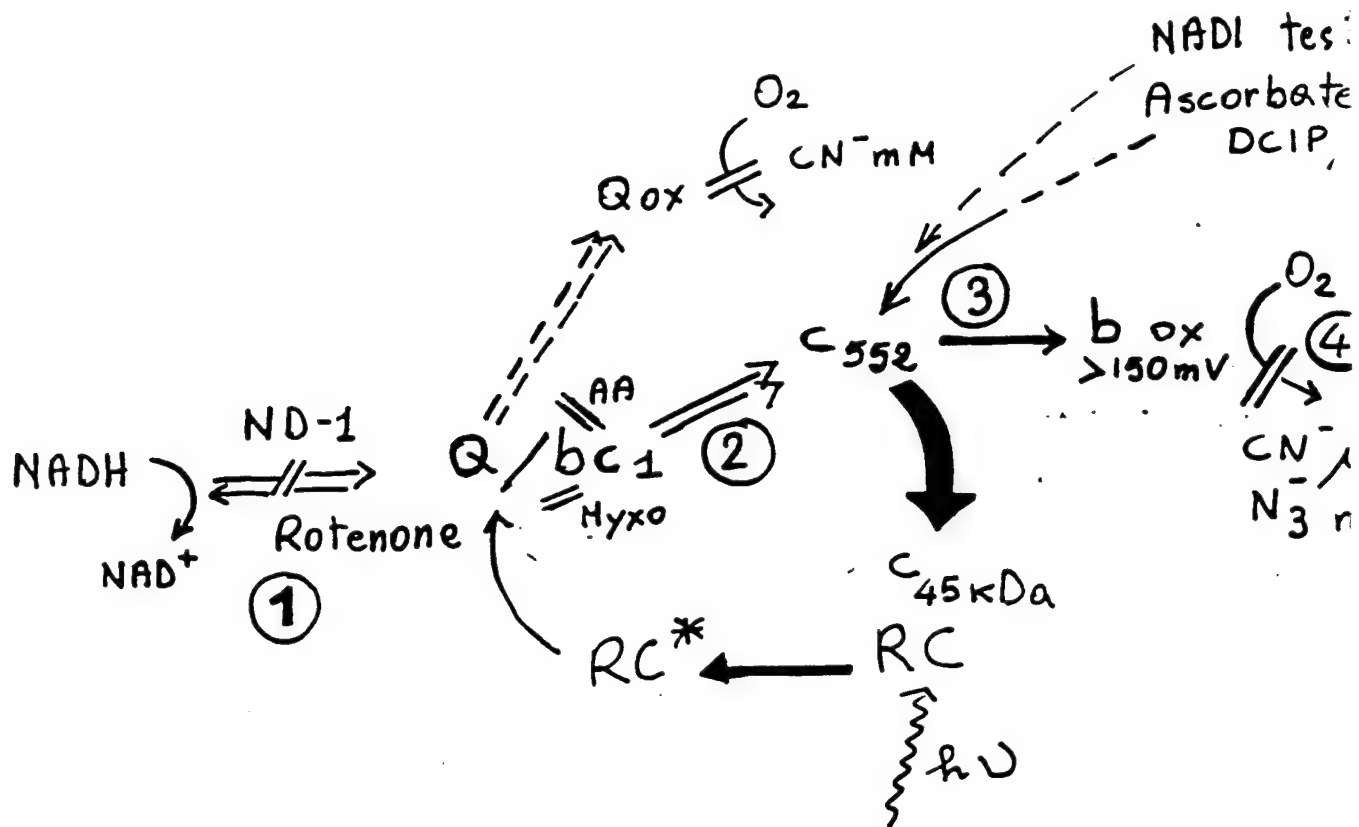
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*Rsp. centenum* redox chain  
D. Zannoni 7/27 '95 WH

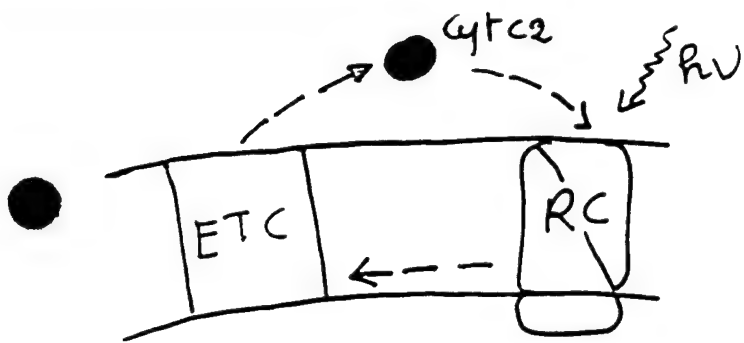
Redox steps affecting positive phototaxis

- ① Slight inhibition
- ② Complete inhibition
- ③ Slight inhibition
- ④ Stimulation

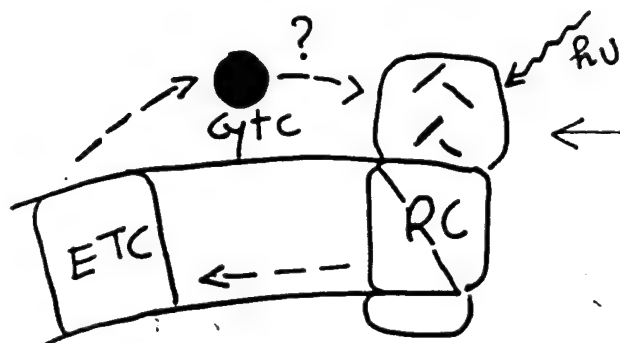
CONCLUSION

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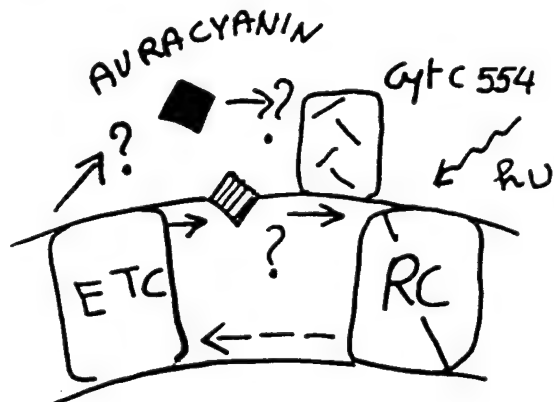
Respiration through the cytochrome oxidase pathway inhibits *Rsp. centenum* phototactic movement.



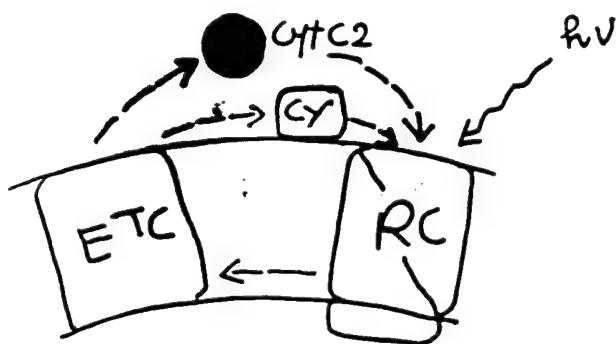
*Rb. sphaeroides*  
*Rsp. rubrum*



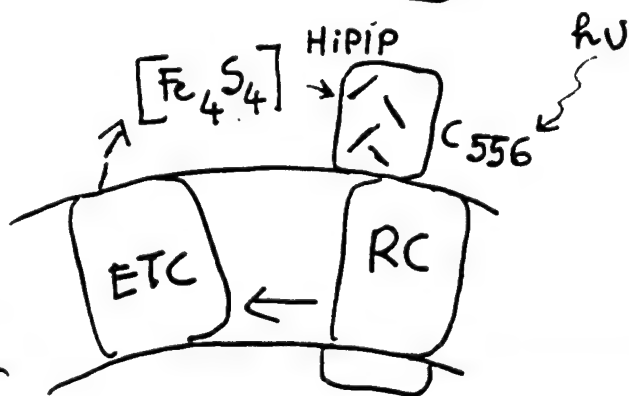
*Rsp. centenum*  
*Rps. viridis*



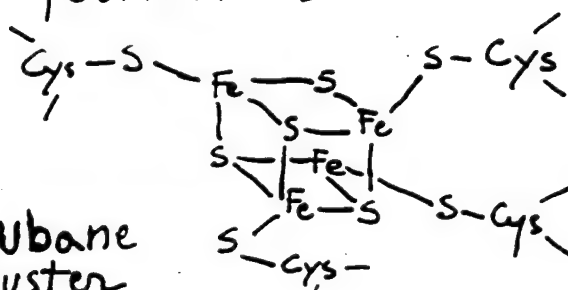
*Chloroflexus aurantiacus*

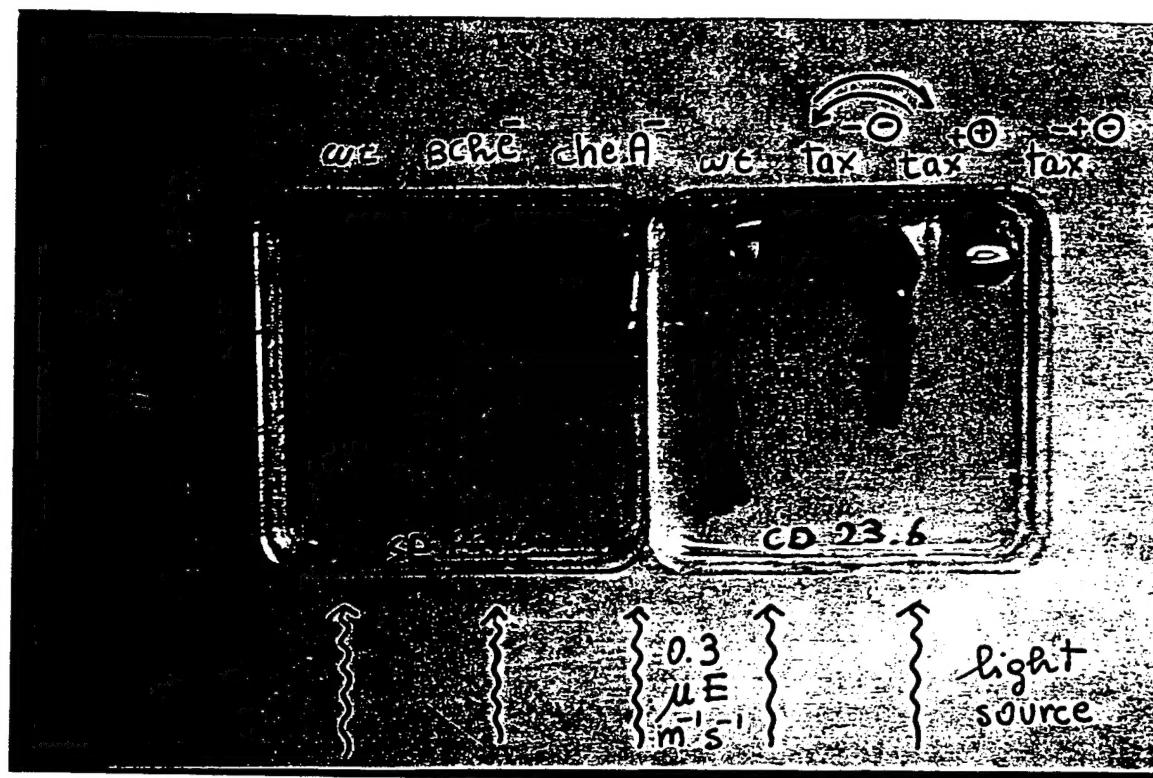


*Rb. capsulatus*



*Rhodospirillum rubrum*



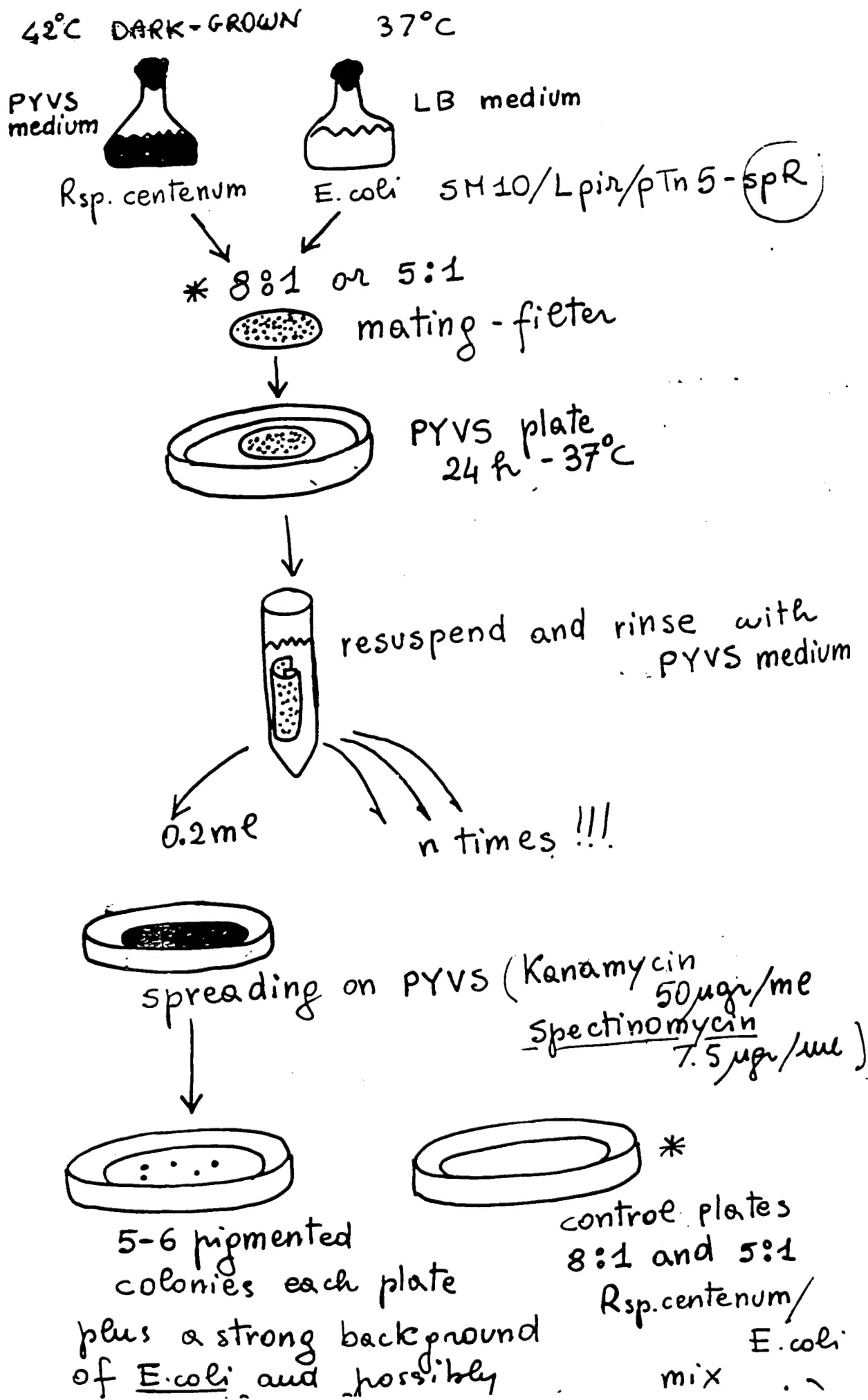


### Legend

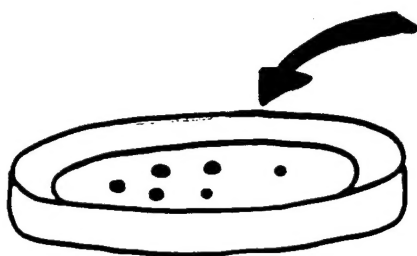
- wt, *Rsp. centenum* wild type
- 707, Bchl deficient mutant
- ZY5, cheA deficient mutant
- YB 2-10, deficient in negative taxis
- YB 77-6, deficient in positive taxis
- YB 271-2, deficient in both positive and negative taxis

Note: labels of 77-6 and 271-2 mutants were mixed up.

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NADH-reaction

( $\alpha$ -naphthol + DMPD)

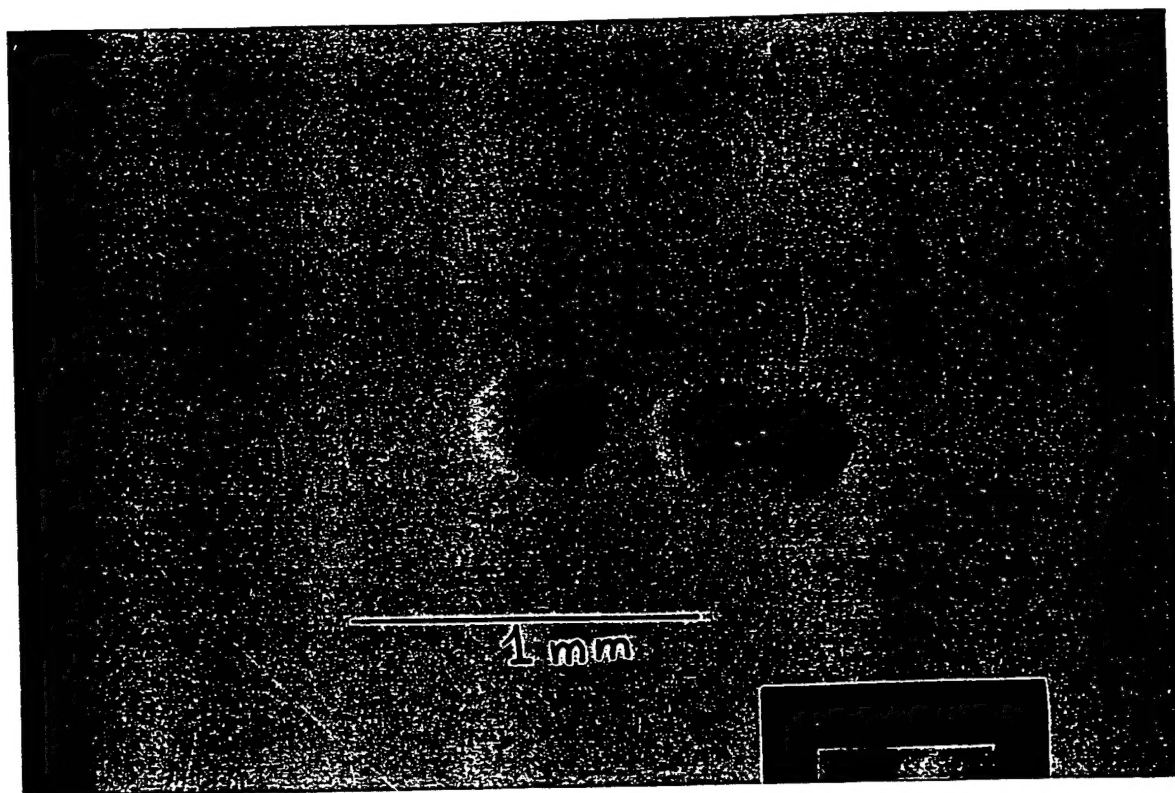
avoid blue colonies and pick up  
(if any) pigmented brown-red-pink  
colonies (only 2!!!) showing NADH<sup>+</sup>



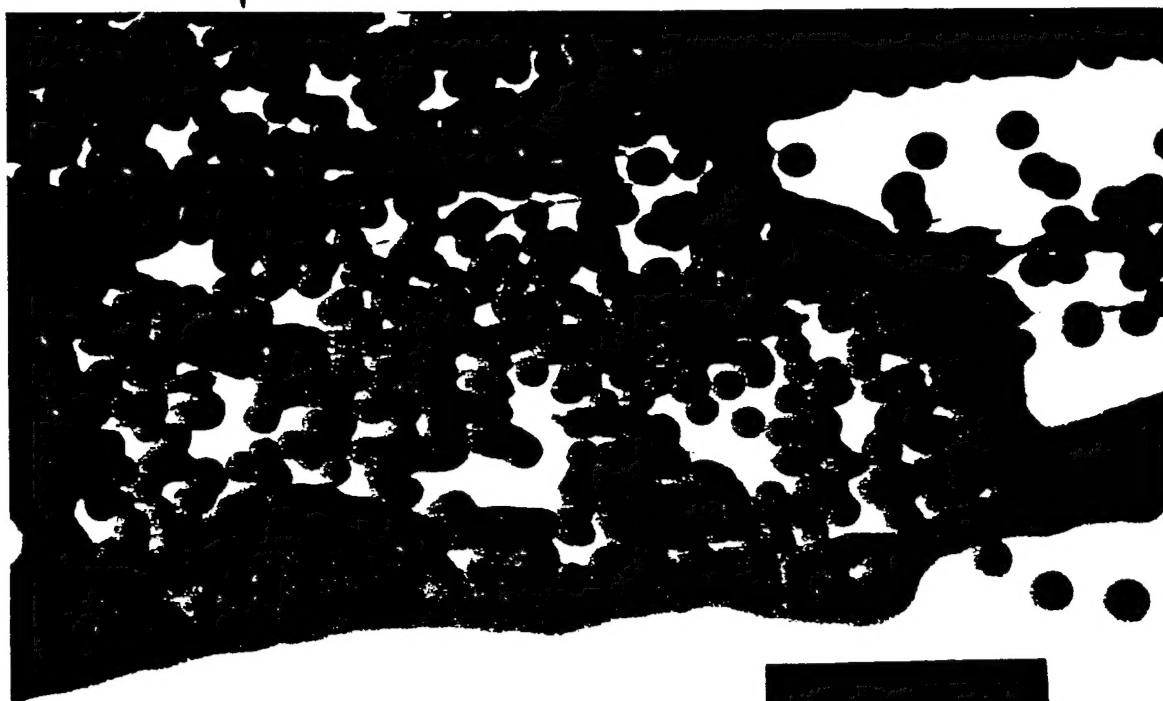
streak them out  
on CENMED plates (Km 50, Sp 7.5)



That's it !!



Tn 5 mutagenized colonies of  
Rsp. centenum (KmR + SpR)



Note: the actual color of the colonies  
was red-pink

DZ NAD<sup>+</sup> mutant (24 h after NAD<sup>+</sup> addition)